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IMMUNOLOGICAL RELATIONSHIP BETWEEN THE VIRUSES OF JAPANESE B ENCEPHALITIS AND JAPANESE EQUINE ENCEPHALOMYELITIS

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The available data regarding immunological relationship of virus strains isolated from Japanese B encephalitis and Japanese equine encephalomyelitis cases are extremely limited.

Sabin[1] performed complement fixation tests on a neurotropic virus isolated from the brain of a fatal case during the 1945 epidemic on Okinawa. The antigen prepared from this Okinawa strain by the freezing and thawing method fixed complement in the presence of mouse immune serum against Japanese B (Nakayama) virus in a titer of 1:512.

Sabin et al[2] reported the results of complement fixation tests of a strain isolated from a fatal case which occurred among United States military personnel in Korea in 1946. The Korea virus antigen prepared by high speed centrifugation fixed complement in the presence of the Japanese B (Nakayama) immune mouse serum to almost the same extent as the known Japanese B (Nakayama) antigen.

There are also few reports in the Japanese literature of use of intracerebral neutralization tests and resistance tests in immune mice as a means of identifying various strains of Japanese equine encephalomyelitis virus.

Kii et al[3] isolated 4 strains from equine cases succumbing to an encephalitis-like infection and compared these strains with Japanese B (Kalinina) virus. In neutralization tests all the strains demonstrated similar antigenic properties. These authors also reported on a strain isolated in 1941 from an equine case of encephalomyelitis.[4] They compared this strain with another equine strain by means of neutralization tests using immune serums against Japanese B virus (human) and the new strain. Both strains exhibited comparable neutralizing properties.

In a previous publication[5] it was reported that Japanese equine encephalomyelitis was caused by a virus immunologically identical with that of Japanese B encephalitis in human patients.

While pursuing epizootic studies of this disease which occurred in Japan during the summer and fall of 1947, two equine strains (no. 2683 and 2869) were isolated. During the epizootic many clinical cases of Japanese B encephalitis were observed among human beings in

Received for publication May 6, 1950.

1. Sabin, A. B. 1947, Epidemic encephalitis in military personnel. J. A. M. A. **133**: 281–293.
2. Sabin, A. B., Schlesinger, H. W., Ginder, D. R. and Matumoto, M. 1947, Japanese B encephalitis in American soldiers in Korea. Am. J. Hyg. **46**(3): 356–375.
3. Kii, N., Ando, K., Sato, K., Okubo, K., Nakayama, T., Ichikawa, S. and Yamada, N. 1937, Etiological studies on epidemic equine encephalitis in Japan. Zikken Igaku Zassi, **21**(2): 117–146.
4. Kii, N., Kuchii, T. and Kitaoka, N. 1942, On epidemic encephalomyelitis occurring at Syonai, Yamagata Prefecture (Japan), 1941. Rikugun Zyui-Danpo, **400**: 1203–1217.
5. Burns, K. F., Tigertt, W. D. and Matumoto, M. 1949, Japanese equine encephalomyelitis in 1947. II Serological and etiological studies. Am. J. Hyg. **50** (1): 27–45.

the same geographical areas, and three strains (2285, 2311 and 2338) were isolated from fatal human cases by this laboratory. The immunological relationship of these equine strains to that of the Nakayama strain of Japanese B encephalitis is striking.

Because of the minute amount of study previously afforded the relationship between various epizootics and human disease in Japan, it is with considerable interest that we present an immunological comparison between the equine isolates, the three human isolates and that of the Nakayama strain of Japanese B encephalitis. This is particularly so in view of the supporting and accumulating evidence which implicates horses as possible "reservoirs"* of the virus in this area.

For convenience, a summary of data on equine isolates (2683 and 2869) which was presented in a previous publication[5] is included in this report.

Isolation of human strains 2285, 2311 and 2338†

These three cases were clinically diagnosed as Japanese B encephalitis. Case 2285 was reported from Kochi Prefecture, located on Shikoku Island of of the Japan Archipelago. The other two cases (2311 and 2338) occurred in Hyogo Prefecture, which is in the southern part of Honshu, the main island of Japan. Sections of brain tissue were forwarded in ½ glycerin saline mixture to this laboratory and were inoculated in mice within 72 hours of death. In each instance the virus was readily recovered.

Case 2285.—On 2 September 1947, brain tissue was triturated in a mortar to a 10% suspension in 10% inactivated rabbit serum in saline, then centrifuged at low speed and the supernatant injected intracerebrally in 0.03 cc amounts into each of 10 white mice, approximately 4 weeks of age. Four of them died within 24 hours and were discarded. Another one showed definite signs of involvement of the central nervous system 8 days after inoculation and was sacrificed for passage. A 10% brain suspension was made from this material, and the supernatant after low speed centrifugation was injected intracerebrally into 10 additional mice, 4 weeks of age. One mouse was found dead 3 days after inoculation, and 4 other mice were obviously ill on the same day, showing ruffled fur, hunching, tremors and paralysis. These 4 mice were used for further passage. The remaining 5 mice of the 2nd passage died on the 6th day with definite central nervous system symptoms. Additional intracerebral serial passages using a 10% infected brain suspension were successfully conducted. One mouse of the 1st passage became ill on the 12th day and died the following day. The remaining 4 mice of this group remained apparently normal during the subsequent 3-week period of observation.

Case 2311.—Glycerinated brain tissue was ground in a mortar to a 10% suspension using as a diluent 10% normal rabbit serum in saline. After low speed centrifugation the supernatant was inoculated intracerebrally (0.03 cc) into 10 mice, 4 weeks of age. Six days subsequent to inoculation, 3 of them were found dead and were discarded because of cannibalism of the entire cranial cavity. Another mouse showed definite signs of encephalitis on the same day and was sacrificed. The second passage with this material was performed as usual, inoculating 0.03 cc intracerebrally of a 10^{-1} brain suspension into each of 10 mice. One mouse of this group died within 24 hours and was discarded. Five mice portrayed symptoms of encephalitis 3 days subsequent to injection, and their brains were removed and used as passage material. Further passages were carried out with ease, the average observed incubation period being 4 to 5 days. The remaining 4 mice of the second passage died 5 days after inoculation with typical central nervous system symptoms. Three of the remaining mice of the first passage demonstrated encephalitic signs and died 8 days after injection, with the last 3 of this group expiring in the same manner on the 10th day.

Case 2338.—Sections of brain tissue preserved in a glycerin saline mixture were ground in a mortar as usual and made to a 10^{-1} concentration,

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* Reservoir animal is used in this paper in a restricted sense. No true reservoir of the virus of Japanese B. encephalitis is as yet known. Such a reservoir, if its exists, might be vertebrate or invertebrate.

† Regarding isolation of equine strains (2683 and 2869), see previous publication.[5]

using as a diluent 10% normal rabbit serum in saline. After low speed centrifugation the supernatant was inoculated intracerebrally into 10 mice. One mouse died 4 days after inoculation, showing no signs of encephalitis, and was discarded. Another mouse demonstrated encephalitic signs and died on the 6th day. Four mice died 7 days after inoculation with typical central nervous system symptoms. On the same day, one mouse was definitely ill and was sacrificed for passage. The second passage with this material was performed as usual into 10 mice. Three of them died 4 days after inoculation. The remaining 7 mice became prostrate on the same day and were sacrificed, the harvested brains being used as additional passage material. In all cases the passage mice showed ruffling of fur, convulsions, tremors and ataxia after an incubation period of 4 to 5 days.

Complement fixation test

Methods and materials.—Complement fixation tests were carried out as described before,[6] with undiluted antigens and twofold serial dilutions of immune serums which were heated at 56 C (guinea pig serum) or 60 C (mouse serum) for 20 minutes. Antigens of Nakayama, 2285, 2311, 2338 and equine isolates 2683 and 2869 strains, St. Louis virus and normal mouse brain were prepared by the freezing and thawing method. A 10^{-1} suspension was prepared by emulsifying the brain tissue of infected mice or normal mice using as a diluent 2% heated normal guinea pig serum in saline. This material was then stored in a refrigerator overnight at 5 C. The following morning the brain suspension was centrifuged at 2000 r.p.m. for 30 minutes; then the supernatant was pipetted off and placed in lusteroid tubes and suspended in a mixture of ethanol and solid carbon dioxide until frozen. The tubes were then transferred to a water bath at 37 C and thawed. This procedure was repeated 4 or 5 times until precipitation occurred. The suspension thus treated was centrifuged horizontally at 2000 r.p.m. for 20 minutes to sediment larger precipitates and then at 18,000 r.p.m. for one hour in a reinforced type SB "International" centrifuge with multispeed attachment refrigerated by dry ice. To the supernatant, merthiolate was added in a final dilution of 1:10,000, and this antigen was stored in an electric refrigerator. The antigen of Western equine encephalomyelitis was supplied by Dr. W. M. Hammon, of the University of Pittsburgh and was prepared by his method of benzol extraction.[6,7] It should be noted that mouse brain antigens do not give nonspecific complement fixation reactions with guinea pig serum, a reaction which occurs very frequently with mouse brain antigens and normal serums from other species (cf. Casals and Palacios[8] and Hammon and Espana.)[9]

Immune serums for Japanese B (Nakayama) and Western equine viruses were prepared in guinea pigs and that for St. Louis virus in mice. For the Nakayama strain of Japanese B encephalitis guinea pigs were immunized as follows: 0.1 cc of 10^{-2} dilution of mouse brain suspension infected with Nakayama strain was injected intracerebrally into normal guinea pigs two times at weekly intervals. Ten days following the last dose, the animal was bled from the heart under ether anesthesia, the serum collected and separated in the usual manner and stored in a refrigerator without added preservative.

Mice used in preparation of immune serums for the St. Louis virus were vaccinated by means of 2 intraperitoneal injections at 2-day intervals of 0.2 cc each of an avirulent formalized vaccine. The vaccine was prepared by injecting intracerebrally sufficient numbers of mice with a 10^{-2} suspension of virus. When prostrate, animals were sacrificed, their brains removed, weighed and emulsified in sufficient saline to give a 10% brain tissue emulsion. Next, 0.2% commercial formaldehyde was added to the brain emulsion and the mixture kept in a refrigerator in glass-stoppered bottles. Usually 10 to 15 days were long enough to inactivate the virus completely. The vaccinated mice were then hyperimmunized by intraperitoneal injection at 5-day intervals with 2 or more 0.5 cc doses of an active 10^{-2} virus suspension of mouse brain tissue. Approximately 10 days after

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6. Espana, C. and Hammon, W. M. 1947, An improved benzene extracted complement fixing antigen for certain neurotropic viruses. Proc. Soc. Exper. Biol. & Med. **66**: 101–102.
7. Espana, C. and Hammon, W. M. 1948, An improved benzene extracted complement fixing antigen applied to the diagnosis of the arthropod-borne virus encephalitis. J. Immunol. **59** (1): 31–44.
8. Casals, J. and Palacios, R. 1941, The complement fixation test in the diagnosis of virus infections of the central nervous system. J. Exper. Med. **74**: 409–426.
9. Hammon, W. M. and Espana, C. 1947, A simple method of producing control guinea pig immune sera for use with complement fixing antigens for the arthropod-borne virus encephalitides. Proc. Soc. Exper. Biol. & Med. **66**: 113–115.

the last dose the hyperimmune mice were bled by heart puncture under ether anesthesia, the serum separated as usual and stored in a refrigerator without preservative.

In order to prepare immune serum of Western equine virus, guinea pigs were injected intraperitoneally with 2 doses 0.5 cc each of a 10^{-1} avirulent formalized (0.2%) vaccine, prepared essentially in the manner described above from infected guinea pig brains. The material was tested for virulence by intracerebral inoculation into young mice, and when it proved to be no longer virulent, it was ready for use. This was followed by 3 additional intraperitoneal injections of 2.0 cc each of a 10^{-2} active guinea pig brain virus. Two weeks after the last dose, the heart blood was collected and allowed to clot. The serum was removed and stored in a refrigerator as previously mentioned.

Results.—Results of complement fixation tests are summarized in table 1.

All antigens of the 2 equine and 3 human isolates, in addition to the Nakayama strain, gave fixation in the presence of Japanese B immune serum, while the antigens of St. Louis virus and normal mouse brain were completely negative. Complement fixation titers for the Nakayama serum in the presence of the Nakayama and 2 equine isolate antigens were at a level of 1:128, while the 3 human isolates fixed complement in a serum dilution of 1:32.

Antigens of human and equine isolates and the Nakayama strain did not fix complement in the presence of St. Louis immune serum, while the homologous antigen had a titer of 1:64.

In the tests using immune serum against Western equine virus, all antigens except that of Western equine virus were negative.

TABLE 1.—*Results of complement fixation tests with hyperimmune animal serums and neurotropic virus antigens.*

Hyperimmune serum	Dilutions of serum	Complement fixation for indicated antigens									
		Japanese B Nakayama	No. 2285*	No. 2311†	No. 2338‡	No. 2683§	No. 2869‖	SLE¶	WEE**	NMB††	Saline
Anti-Japanese B encephalitis (Nakayama) guinea pig serum	1:8	4	4	4	4	4	4	0		0	0
	1:16	4	4	4	4	4	4	0		0	
	1:32	4	3	2	3	4	4				
	1:64	4	±	0	±	4	4				
	1:128	3	0	0	0	3	3				
	1:256	0	0	0	0	0	0				
	1:512	0	0	0	0	0	0				
Anti-St. Louis encephalitis mouse serum	1:2	±	0	0	0	0	0	4		0	0
	1:4	0	0	0	0	0	0	4		0	
	1:8	0	0	0	0	0	0	4			
	1:16							4			
	1:32							4			
	1:64							4			
	1:128							0			
Anti-western equine encephalomyelitis guinea pig serum	1:2		0	0	1	±	0		4	0	0
	1:4		0	0	0	0	0		4	0	
	1:8		0	0	0	0	0		4		
	1:16		0	0	0	0	0		4		
	1:32		0	0	0	0	0		0		
	1:64		0	0	0	0	0		0		

Complete fixation is regarded as 4; different degrees of partial fixation as 3, 2 or 1; questionable trace as ±; no fixation or complete hemolysis as 0.

* Fourth passage.
† Fifth passage.
‡ Fourth passage.
§ Fifth passage.
‖ Second passage.
¶ St. Louis Encephalitis.
** Western equine encephalomyelitis.
†† Normal mouse brain.

Neutralization test

Methods and materials.—Neutralization tests were performed as described in a previous publication[8] with equal amounts of undiluted, unheated serum and serial 10-fold dilutions of mouse brain suspension of the virus in question. These serum-virus mixtures were incubated in a

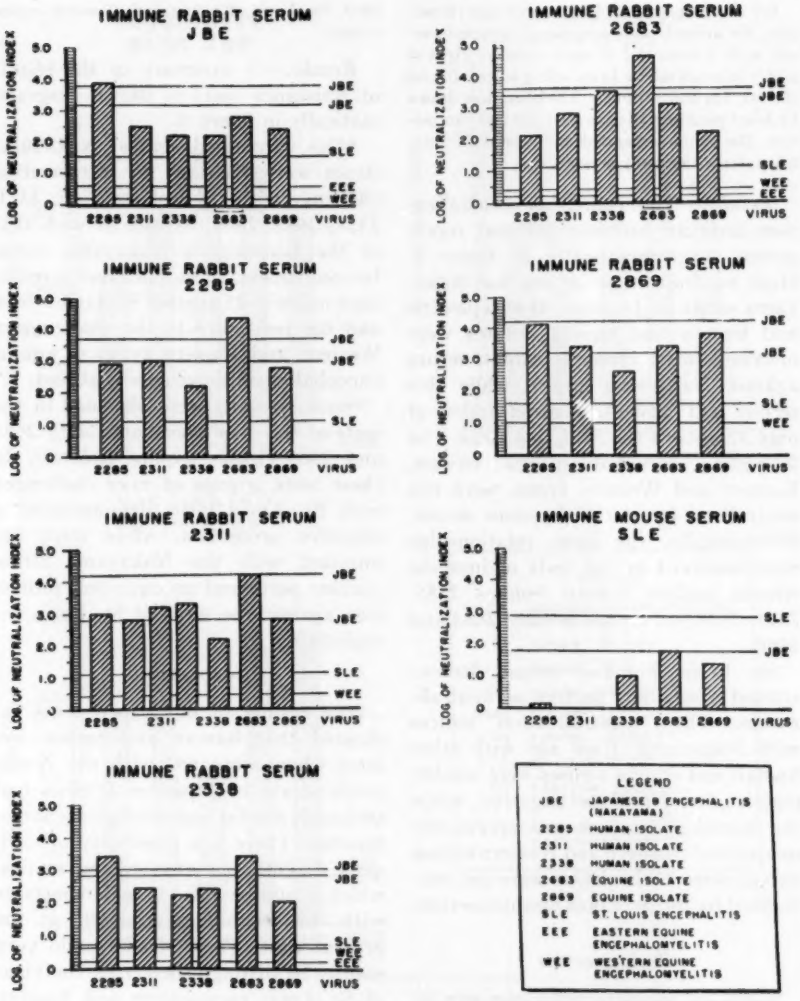

FIG. 1.—Cross neutralization tests.

water bath at 37 C for two hours and then inoculated intracerebrally into 4-week-old mice, each mouse receiving an inoculum of 0.03 cc. Five mice were used per dilution of the virus suspension. Inoculated mice were observed for 2 weeks and the survivors tabulated. Mice which died within 3 days after inoculation were disregarded. Normal rabbit serum known to be negative was included as control in each test. The 50% end point was calculated as usual, and the neutralization index was obtained as an antilog of the difference in log dilutions of LD_{50} end points of virus-serum mixtures between the normal rabbit serum control and immune serum. No serum was regarded positive unless the index was 50 or greater.

Immune serums used in neutralization tests were prepared in rabbits except that for the St. Louis virus, which was prepared in mice in the same manner as outlined for the complement fixation tests.

For the preparation of immune serum in rabbits, the animals were inoculated intraperitoneally with 3 doses of 5 cc each of active virus of a 10^{-1} infected mouse brain saline suspension on the 1st, 5th and 12th day. The blood was drawn by heart puncture 10 days after the last vaccination, the serum separated and stored in a refrigerator without any preservative.

Results.—The results of neutralization tests are summarized and represented diagrammatically in figure 1. High neutralization indices for Nakayama strain of Japanese B encephalitis and human and equine isolates were obtained with immune rabbit serum against Nakayama strain, while this serum had a neutralization index of only 32 against the St. Louis virus. The 2 equine encephalomyelitis viruses, Eastern and Western types, were not neutralized by the Nakayama serum. Substantially, the same relationships were observed in the tests of immune serums against human isolates 2285, 2311, 2338 and equine isolates 2683 and 2869.

St. Louis immune serum demonstrated a different pattern of neutralization. Thus, neutralization indices with Nakayama strain and with other human and equine viruses were weakly positive, equivocal or negative, while the homologous virus was specifically neutralized. Eastern and Western equine encephalomyelitis viruses were not neutralized by the St. Louis immune serum.

Resistance test

Methods and materials.—White mice were immunized with the Nakayama strain of Japanese B encephalitis and with two equine isolates. In order to immunize these animals, 2 intraperitoneal inoculations of 0.5 cc each of a formalized (0.2%) vaccine prepared from 10% infected mouse brain were given at 2-day intervals. This was followed with 2 doses of 0.5 cc each of 10^{-2} dilution of active viruses at 7-day intervals. These vaccinated mice were challenged intracerebrally 2 weeks following the last dose of vaccine with Nakayama, 2285, 2311, 2338, 2683, 2869, St. Louis, Eastern and Western equine viruses.

Results.—A summary of the results of resistance tests is shown diagrammatically in figure 2.

Mice vaccinated against Nakayama strain were resistant to intracerebral challenge with the isolates 2285, 2311, 2338, 2683, 2869, as well as with that of the homologous Nakayama virus. In comparison, these mice had a resistance index of 25 against St. Louis virus, and the resistance to the challenge of Western and Eastern types of equine encephalomyelitis virus was absent.

Similar results were obtained in the tests of the mice vaccinated with 2683 and 2869 viruses (equine isolates). In these tests, groups of mice challenged with St. Louis virus demonstrated a negative protection, while mice immunized with the Nakayama strain vaccine portrayed an equivocal protection against the virus of St. Louis encephalitis.

DISCUSSION

The results of our experiments indicated that human and equine isolates when compared with the Nakayama strain of Japanese B virus had strikingly similar immunological characteristics. There is a possibility of antigenic dissimilarity among these strains, which would be difficult to demonstrate with the techniques available at the present time. These strains could very readily be distinguished from the virus of St. Louis encephalitis and Eastern and Western types of equine encephalomyelitis.

In the complement fixation tests, these six strains fixed complement in the presence of Japanese B (Nakayama) immune guinea pig serum but exhibited no fixation in the presence of St. Louis immune mouse serum and Western equine immune guinea pig serum. The

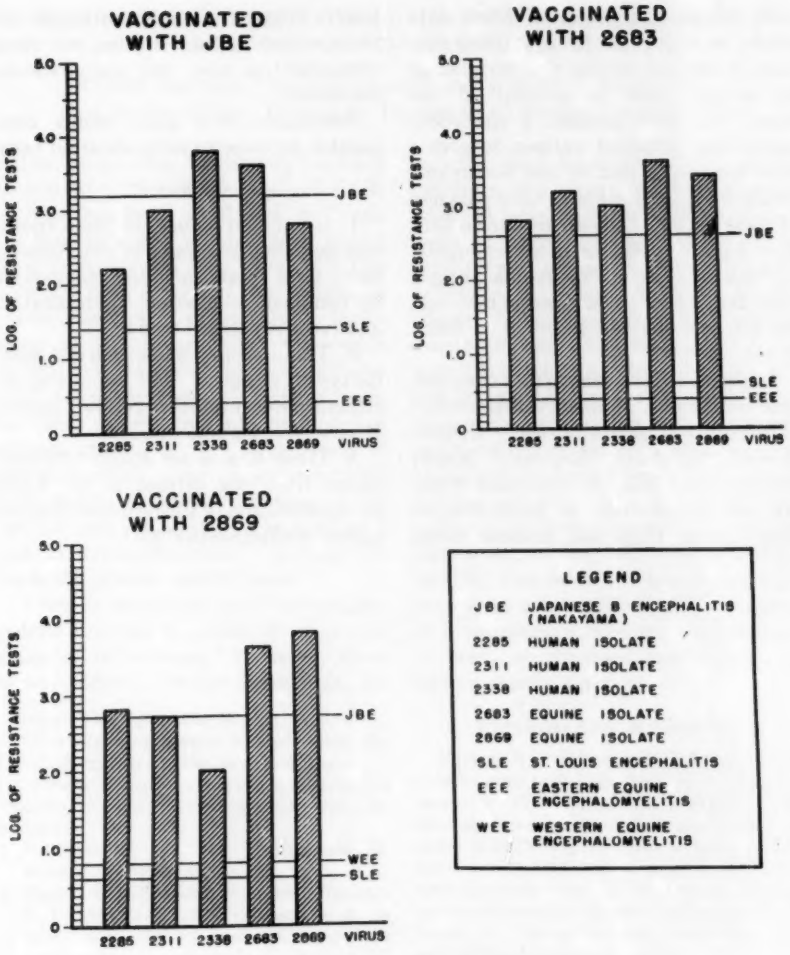

FIG. 2.—Cross resistance tests.

specificity and the potency of these serums were quite evident as shown in results obtained with homologous antigens.

As shown in table 1, the complement-fixing titers with the antigens of our 3 strains (2285, 2311 and 2338) were lower than those of the Nakayama control, results which compare with those of Sabin.[1] Antigen prepared from mouse brain of the 4th passage of 2311 gave only a slight fixation of complement in serum dilution of 1:8. The 5th passage antigen gave a 1:32 serum dilution titer as shown in table 1. A similar result was observed with 2683 isolate, which demonstrated a negative fixation with the 2nd passage and became positive

with 5th passage material. These data might be explained by low concentration of antigen during the early stage of mouse brain adaptation of the virus. In fact, antigen preparations containing sufficient antigen to give a titer similar to that of the Nakayama strain were obtained with later passages of these strains. In contrast, strain 2869 gave a positive fixation in a titer equally as high as that of Nakayama antigen with 2nd mouse brain passage material. Such a case was also observed by Sabin et al.[2]

In the neutralization tests, a considerable degree of immunological similarity of these six strains was clearly demonstrated. Moreover, they were readily distinguished from St. Louis and Western and Eastern equine encephalomyelitis viruses. There are, however, some results suggesting slight antigenic differences among these strains, but these dissimilarities were not unequivocally confirmed.

Resistance tests gave results comparable to those of neutralization tests.

SUMMARY

1. Continued study of two equine and three human strains of virus isolates from fatal cases included comparison by complement fixation, neutralization and cross resistance tests.

2. These strains show marked similarity of antigenic and immunogenic structure to one another and to Japanese B virus (Nakayama strain).

3. These strains are readily differentiated from the viruses of St. Louis encephalitis and of Eastern and Western equine encephalomyelitis.

ERYTHROCYTE DESTRUCTION IN TUBERCULOUS ANIMALS FOLLOWING TUBERCULIN INJECTION

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The low-grade or "secondary" anemia associated with clinical tuberculosis in humans has been attributed to "both the bacillary and non-bacillary toxins from broken-down focal tissues."[1] The view has been expressed that anemia in tuberculosis, as in other chronic infections, is due to an interference with normal hematopoietic functioning,[2] and that tuberculoprotein interferes with synthesis of hemoglobin.[3] The inclusion of such anemia in "the large group of anemias which occur as an associated symptom with some other disease—(and which)—are probably for the most part due to dyshaemopoiesis"[4] perhaps reflects the present general view.

There is no doubt but that hematopoietic function is inhibited in certain types of tuberculosis.[5] However, there is no evidence, to our knowledge, indicating that tuberculoprotein per se possesses this capacity. In the work reported here, however, tuberculin was found to have a profound effect on the erythrocytes of hypersensitive but not on those of normal animals. In the course of investigations on the mechanism of the tuberculin reaction, we noted that blood taken from tuberculous rabbits and guinea pigs during mild tuberculin shock appeared to have undergone extensive hemolysis. Results of hematological studies have subsequently shown (a) that following the injection of tuberculin, a rapid destruction of erythrocytes does occur in the tuberculous animal, and (b) that such erythrocyte damage may occur continually during the course of a progressive tuberculosis infection, without an obvious impairment of hematopoietic function.

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1. Pottenger, F. M. 1948, Tuberculosis, St. Louis, C. V. Mosby Co., p. 303.
2. Osgood, E. E., Haskins, H. D. and Trotman, F. E. 1932, The value of accurately determined color, volume and saturation indexes in anemias; based on study of over 200 patients. J. Lab. & Clin. Med. **17**: 859.
3. Doan, C. A., Wiseman, B. K. and Curtis, G. M. 1935, A symposium: Progress in tuberculosis. J. Ohio State Med. Soc. **31**: 926.
4. Whitby, L. E. H. and Britton, C. J. C. 1947, Disorders of the Blood, Phila., The Blakiston Co., p. 147.
5. Doan, C. A. and Sabin, F. R. 1927, Local progression with spontaneous regression of tuberculosis in the bone marrow of rabbits, correlated with a transitory anemia and leucopenia after intravenous inoculation. J. Exper. Med. **46**: 315.

EXPERIMENTAL PROCEDURE

White male rabbits weighing 3.5 to 4 kg each were infected with large doses (4 to 5 mg wet weight) of the Ravenel strain of *Mycobacterium tuberculosis* var. *bovis*. A few rabbits were infected with BCG. Five weeks or more were allowed between injection of tubercle bacilli and use in the experiments. All the animals, therefore, had developed a high degree of skin sensitivity to tuberculin. Control animals, uninfected, were selected from the same shipment.

Blood samples (usually 5 ml) were taken from each rabbit by cardiac puncture while the animal was rigidly secured to an operating board. No anesthesia was used. The ammonium and potassium oxalate mixture of Heller and Paul[6] was used as anticoagulant. Immediately following bleeding, the animals were injected intravenously (marginal ear vein) with tuberculin. PPD was

6. Heller, V. G. and Paul, H. 1934, Changes in cell volume produced by varying concentrations of different anticoagulants. J. Lab. & Clin. Med. **19**: 777.

used exclusively, in a concentration of 1.0 mg per ml of buffered diluent.

At intervals following tuberculin injection, additional blood samples were taken. All erythrocyte counts were made by one person. Hematocrit determinations were made in Wintrobe hematocrit tubes. A Klett-Summerson photoelectric colorimeter was used for hemoglobin determinations. All such work was done within an hour of bleeding.

RESULTS

In table 1 are shown data typical of those obtained when PPD was injected into rabbits with active tuberculosis. The precipitous decrease in erythrocyte count (animal 328) 20 hours after tuberculin injection was accompanied by a radical increase in mean corpuscular volume (M.C.V.). Forty hours later, compensatory red cell production had begun and the M.C.V. had returned to normal. The reaction of animal 838, while differing in rate, was similar, i.e., as the number of red cells decreased, the M.C.V. rose and then returned to normal.

The data in table 2 demonstrate differences in rate of M.C.V. change which appear to be related to the stage and kind of infection present. Animal 7 had active, progressive tuberculosis, was losing weight, and its erythrocyte count had decreased by almost a million since it had been infected. It will be noted that the M.C.V. was 83.3 cubic microns or approximately 25% above normal for this animal. We have found that such a high M.C.V. is observed very frequently in progressive tuberculosis in rabbits. In such cases, a further increase in M.C.V. following injection of tuberculin may or may not be apparent, depending upon when blood samples are taken.

Animal 49, although hypersensitive, did not have a progressive infection. Its M.C.V. at the time of injection of tuberculin was near its normal level, and the quantity of tuberculin injected caused a relatively slight increase. In such animals sensitized with BCG it is necessary to use much larger quantities of tuberculin to produce marked changes in the blood picture.

Figure 1 summarizes the results obtained in one experiment over a three-

TABLE 1.—*Blood changes observed in rabbits during 90 hours following intravenous injection of 0.5 mg of PPD.*

Hours after PPD injection	Normal no. 12				No. 328*				No. 838*			
	RBC	Hemato-crit	M.C.V.	Hemo-globin	RBC	Hemato-crit	M.C.V.	Hemo-globin	RBC	Hemato-crit	M.C.V.	Hemo-globin
0	5.08	36.0	71.0	10.40	4.07	30.2	74.2	8.8	4.29	33.0	76.9	9.2
12	4.98	34.6	69.5	10.15	3.55	28.0	78.5	7.7	3.88	31.0	80.0	9.0
20	4.68	33.3	71.5	9.75	1.98	19.8	100.0	5.8	3.74	32.2	86.0	9.0
38	4.60	32.2	70.0		2.19	18.9	87.0		3.80	35.5	93.4	
62	4.95	35.7	72.0	10.30	2.74	18.7	68.2	6.4	2.45	18.2	74.2	6.2
90	5.00	35.0	70.0	10.60	3.20	22.3	69.8	7.0	3.50	25.2	72.0	8.2

* Six weeks after subcutaneous injection of 1.0 mg/kg of tubercle bacilli (Ravenel).

TABLE 2.—*Blood changes observed in rabbits during 90 hours following the intravenous injection of 0.2 mg of PPD.*

Hours after PPD injection	Normal no. 30				No. 7 (Ravenel)*				No. 49 (BCG)†			
	RBC	Hemato-crit	M.C.V.	Hemo-globin	RBC	Hemato-crit	M.C.V.	Hemo-globin	RBC	Hemato-crit	M.C.V.	Hemo-globin
0	5.41	37.0	68.4	10.3	4.32	36.0	83.3	9.7	5.12	38.5	76.2	11.1
18	5.38	36.0	66.7	10.3	3.80	33.1	87.1	9.2	4.81	39.6	82.5	10.3
36	5.20	35.0	67.5	8.7	3.53	26.0	73.6	6.5	4.46	37.0	82.9	9.7
56	4.95	33.2	67.0	8.8	5.37	41.2	76.7	10.6	4.62	33.6	72.9	
90	4.80	32.6	68.0	9.3	5.27	35.2	66.7	9.3	5.33	39.0	73.1	11.4

* Five weeks after subcutaneous injection of 1.0 mg/kg of tubercle bacilli (Ravenel).
† Seven weeks after subcutaneous injection of 4.0 mg/kg of BCG.

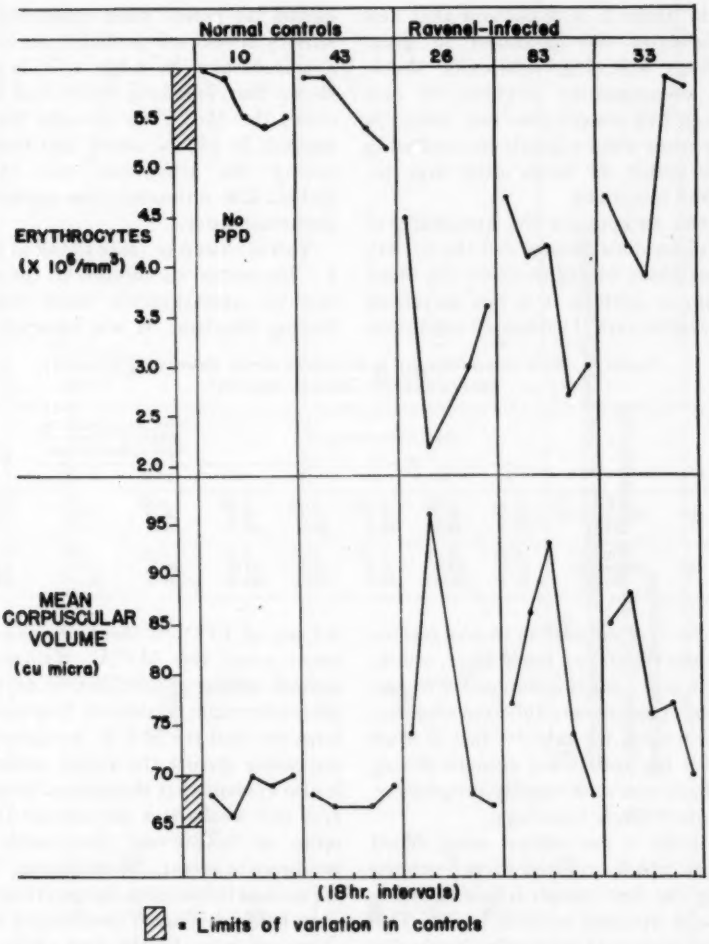

FIG. 1.—Effect of intravenous injection of PPD (0.5 mg) on erythrocytes of rabbits.

day period. A comparison of columns 1 and 2 shows that the injection of tuberculin into normal animals produces no greater blood changes than those resulting from handling and bleeding. The tuberculous animals were in a relatively early stage of infection, were somewhat anemic, and had M.C.V. above normal. Following the intravenous injection of 0.5 mg of PPD, the M.C.V. increased rapidly as the cell count dropped. It will be noted that the minimum M.C.V. after 72 hours falls within the normal range, which indicates that the high values observed prior to tuberculin injection were actually abnormal for each of the animals.

There is no evidence that hematopoietic function is inhibited by tuberculin injection. In tables 1 and 2 it may be seen that hemoglobin values roughly parallel red cell counts. In these tables

and in figure 1, it is obvious that new erythrocytes are produced in great numbers following tuberculin shock, This compensatory erythrocyte production was always observed, except in a few cases when animals succumbed to shock within 48 hours after large tuberculin injections.

When we consider the complexity of the tuberculous process and the variety of conditions which can affect the blood picture in rabbits, it is not surprising that results vary. Under such conditions it can be very misleading to pool results. Since the rabbit is a fairly large, sturdy animal, and since it is impossible to predict the exact course a tuberculosis infection will take, we have studied in some detail a few individual animals during the entire course of rapidly progressive, ultimately fatal, infections.

In table 3 are shown some blood changes which occurred in two rabbits during the first month following infection with virulent tubercle bacilli. This general picture of gradually developing anemia and gradually increasing M.C.V. is one which we have observed repeatedly. During the early period of infections, it is particularly evident. Even when the results were averaged, in one group of six young rabbits the erythrocyte count dropped from 5.3 to 4.4 million per mm³ and the M.C.V. increased from 74.0 to 84.5 cubic microns between the 10th and 20th day following injection of virulent bacilli. The fact that these changes take place during the period when tuberculin sensitivity develops is probably not simply a coincidence. In table 3, it is again shown that, following injection of tuberculin, the M.C.V. is brought back to normal. It will be noted also that following this treatment, the M.C.V. (rabbit 438) ultimately rose again to an abnormal value.

This is shown in more detail in figure 2. This animal succumbed to the infection in approximately three months. During this time, it was injected with 0.2 mg of PPD on three occasions. In every case, the M.C.V. returned to normal within approximately 48 hours after tuberculin treatment. It is evident, however, that the M.C.V. was gradually increasing during the entire period. It is also evident that this animal after the first two weeks was not anemic in the sense of having an abnormally low erythrocyte count. Nevertheless, there are several indications that erythrocytes were being destroyed continually at an abnormal rate. In the first place, this would be expected from the M.C.V. values alone. Secondly, it will be noted that following the second tuberculin injection, the erythrocyte count thereafter remained at a level which was probably higher than normal for this animal, and may represent the response to continuous erythropoietic stimulation. During the last three weeks, there was a gradual decline in cell count, but the presence of hemoglobin in the serum during this time indicates that

TABLE 3.—*Blood changes observed in two rabbits during the course of ultimately fatal tuberculosis (Ravenel) infections.*

		Days following infection					After intravenous injection of 0.2 mg of PPD on the 35th day		After 60 days
		5	12	18	25	31	16 hr.	56 hr.	
No. 441	RBC	5.21	4.58	4.30	4.08	3.82	3.80	3.20	Died 56th day
	Hematocrit	37.0	36.5	35.0	34.5	34.5	30.0	23.4	
	M.C.V.	71.0	79.6	81.5	84.5	90.3	79.0	73.0	
No. 438	RBC	5.33	4.37	3.74	4.13	3.87	3.58	4.05	3.89
	Hematocrit	37.7	35.0	31.8	33.0	33.0	33.2	29.2	34.5
	M.C.V.	70.7	80.0	85.0	80.0	85.4	92.5	72.0	88.6

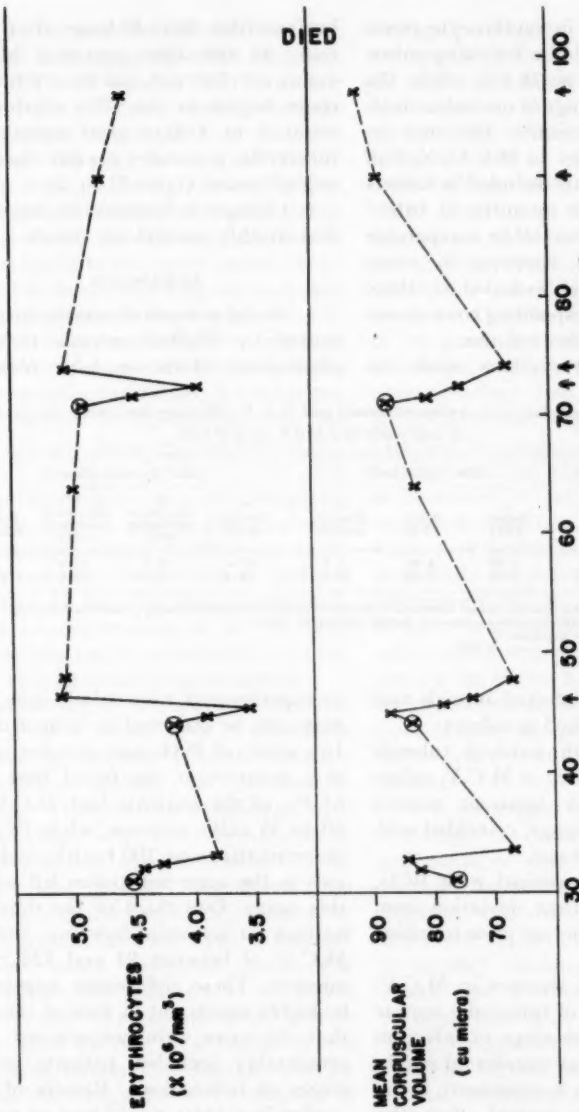

FIG. 2.—Changes in erythrocyte counts and M.C.V. observed in the course of a fatal infection with the Ravenel strain of *Myco. tuberculosis*.

the hematopoietic system was still functioning, probably at an increased rate and against odds. In this case, then, there was no evidence that the capacity to produce erythrocytes was inhibited.

Although we have generally been more interested in observations made in individual animals, inspection of the data in table 4 reveals that there is a significant difference between normal and tuberculous animals even when results are averaged. For example, the

average decrease in erythrocyte count of tuberculous rabbits following tuberculin injection was 28.3%, while the corresponding change in normal animals was only 7.5%. A similar difference appeared with respect to M.C.V. Not all tuberculous animals included in table 4 received the same quantity of tuberculin, nor were they all in comparable stages of infection. However, for every tuberculous animal included in these averages, the corresponding controls are included in the other column.

Results of observations made on approximately 40 infected animals may be briefly summarized as follows:

1. Infection with virulent tubercle bacilli always resulted in M.C.V. values above normal. An apparent anemia usually, but not always, coincided with such changes in cell size.

2. In animals sensitized with BCG, there is no significant deviation from the normal blood picture prior to tuberculin injection.

3. The observed changes in M.C.V. following injection of tuberculin appear to depend upon the stage of infection and/or sensitivity as manifested partly by the M.C.V. prior to treatment. If the original M.C.V. is normal, then the immediate result is an increase. If the M.C.V. is very high before treatment, then a subsequent increase may or may not be apparent (see fig. 1 and 2).

4. Unless lethal quantities of tuberculin are injected, recovery usually begins within 36 to 48 hours after injection. At this time, minimal M.C.V. values are observed, and the erythrocyte count begins to rise. The erythrocyte count 2 to 3 days after injection of tuberculin is usually greater than the original count (table 2, fig. 2).

5. Changes in hemoglobin concentration roughly parallel cell counts.

TABLE 4.—*Average changes in erythrocyte counts and M.C.V. following intravenous injection of tuberculin (0.2 to 0.9 mg of PPD).*

	RBC ($\times 10^6$/mm^3)			M.C.V. (cubic microns)			
	1 Before PPD	2 After PPD	3 Percent decline	4 Before PPD	5 Maximum following PPD	6 Minimum following PPD	7 Percent difference
9 normal animals	5.25	4.86	7.5	67.6	70.5	67.0	5.2
21 Ravenel-infected animals	4.71	3.38	28.3	78.9	88.4	70.9	24.7

Column 2 and 6 = average minimum values observed at any time within 96 hours following tuberculin injection.
Column 5 = average maximum values observed during similar 96 hours.

$$\text{Column 7} = \frac{\text{maximum} - \text{minimum}}{\text{minimum}} \times 100.$$

DISCUSSION

A careful analysis of certain data presented by Muller[7a] reveals that the phenomena which we have observed in experimental tuberculosis infections may also be observed in human cases. In a study of 2584 cases of tuberculosis in a sanatorium, she found that only 55.3% of the patients had M.C.V. of 80 to 91 cubic microns, while 98% of determinations on 100 healthy individuals in the same institution fell within this range. One third of the determinations on patients, however, showed M.C.V. of between 92 and 122 cubic microns. These differences appear to be highly significant in view of the fact that the cases were not selected, and presumably included patients in all stages of tuberculosis. Results of our studies in rabbits would lead us to expect even more significant variations in

7. Muller, G. L. 1943, Clinical Significance of the Blood in Tuberculosis, N. Y., The Commonwealth Fund, (a) pp. 199–207, pp. 212–229; (b) chap. 29 and 30, pp. 217–221; (c). pp. 387–388; (d) pp. 226–229.

M.C.V. between normal persons and patients with progressive, active tuberculosis.

In Muller's data referred to above, it is important to note that the majority of these 2584 patients were not anemic in the sense of having abnormally low hemoglobin values or erythrocyte counts. In her tabulation of 250 tuberculosis patients who were anemic, however, it is shown that 12% had a macrocytic anemia, with an average M.C.V. of 100 cubic microns. Unfortunately, in no tabulated data does Muller indicate the severity of the disease in comparison with the kind or degree of anemia. She does indicate, however, that macrocytic anemia develops in association with hemorrhages in the form of hemoptysis or after surgery such as thoracoplasty. It was also observed following pneumothorax and other kinds of collapse therapy. In one case history recorded, there was an increase in M.C.V. from 77.8 to 106.3 cubic microns immediately following pneumothorax treatment.

Shlomovitz et al[8] concluded that the M.C.V. is a better index of the status of the tuberculosis patient than are hemoglobin values or erythrocyte counts. This view is consistent with present knowledge, however incomplete, of the mechanisms involved in red cell destruction. Ponder[9] has shown that for the erythrocytes of any species, including man, there is a "critical volume" beyond which hemolysis occurs. For the rabbit, this volume is in the region of 105 cubic microns, and we can assume that when the M.C.V. is high, red cells are being destroyed, whether by hemolysis in the circulation or by removal in the spleen. If such a process were occurring continually, anemia need not necessarily be observed. Compensatory cell production could very well balance destruction, up to the point of depletion of nutritive substances required for cell production or until hematopoietic tissue had been exhausted.

The paucity of detailed data on erythrocyte changes in tuberculosis is probably due to the fact that the associated anemia is seldom severe. Furthermore, a multitude of factors contribute to low-grade anemia of infection, and this is particularly true of chronic diseases. Perhaps this accounts for the facts that ordinarily little importance is attached to anemia associated with infections, and that anemia in tuberculosis is presumed to be an incidental, or secondary, condition. It is possible, however, that destruction of erythrocytes is causally related to the existence of hypersensitivity of the tuberculin type. If so, a continuous destruction of red cells during active tuberculosis would place a burden on hematopoietic tissue which, over a long period of time, would have important physiological effects quite apart from clinically apparent anemia.

It is recognized that infections may contribute to anemia by preventing utilization of essential nutritive substances, by a direct toxic action on marrow, and by the production of hemolytic toxins. The possible existence of another mechanism of erythrocyte destruction, perhaps intimately related to hypersensitivity of the tuberculin type, is suggested by our data reported here and by various data on anemia in human tuberculosis.

Because of the complexity of tuberculosis as a pathological entity, and because many of the hematological changes observed in clinical tuberculosis have not been adequately explained, we may be justified in suggesting that some of the clinical symptoms observed

8. Shlomovitz, B. H., Hedding, B. E. and Kehoe, E. J. 1924, Relative blood cell volume in pulmonary tuberculosis. J.A.M.A. **82**: 1845.
9. Ponder, E. 1948, Hemolysis and Related Phenomena, N. Y., Grune and Stratton, pp. 101–109.

are referable to the more or less continuous release into the circulation of substances with tuberculin activity. The destruction of erythrocytes is consistent with the fever observed during the active disease. The extreme reactions which we have produced in rabbits find a counterpart in severe systemic reactions occurring in humans following the injection of large quantities of tuberculin. The same results are often observed following collapse therapy,[7b] which might be expected to squeeze large quantities of tuberculin into the circulation, after which there would be, temporarily at least, a diminution in quantity secreted. It is known that collapse therapy is often followed by rapid changes in red cell volume and numbers,[7c] and we may question the explanation offered by Muller—namely, that these changes represent simply a physiological adjustment to a diminished oxygen supply.

This type of erythrocyte destruction might explain the great irregularity of anemia in tuberculosis, since the presence or absence of anemia would depend upon so many factors. Furthermore, when anemia exists in tuberculosis, it usually responds favorably to iron or liver therapy,[7d] and it is possible that such patients need greater quantities of hematopoietic nutrients because of accelerated blood cell destruction.

The actual mechanism responsible for the erythrocyte destruction during tuberculin shock is a matter for conjecture. Favour[10] has reported that lymphocytes of tuberculous animals are lysed in vitro by tuberculin. Our own observations indicate that destruction of lymphocytes in vivo precedes the rapid decline in red cell numbers, and that the two phenomena may be causally related.[11] We have also found that during tuberculin shock, whole blood of tuberculous animals frequently possesses the capacity to confer tuberculin sensitivity on normal animals.[12] The destruction of red cells is possibly due, then, either to some disintegration product of leucocytes or to a "sensitization" of red cells similar to the process suggested by Miller et al[13,14] to be operative in the case of leucocytes.

10. Favour, C. B. 1947, Lytic effect of bacterial products on lymphocytes of tuberculous animals. Proc. Soc. Exper. Biol. & Med. **65**: 269.

SUMMARY

1. The intravenous injection of small quantities of tuberculin into rabbits with active tuberculosis results in a destruction of erythrocytes, accompanied by significant changes in mean corpuscular volume (M.C.V).

2. Changes in M.C.V. and erythrocyte counts, similar in nature but differing in degree, occur in rabbits during the course of ultimately fatal infections without the injection of tuberculin. It is suggested that such effects are due to liberation of tuberculin from foci of infection.

3. The possibility that a similar process of red cell destruction may operate in human tuberculosis is discussed.

11. Brandt, N., Sandage, C. and Birkeland, J. M. 1950, Effect of intravenous injection of tuberculin on leucocytes of normal and tuberculous rabbits. Proc. Soc. Exper. Biol. & Med. **74**: 215.
12. Sandage, C. and Birkeland, J. M. 1949, Abstracts of papers, 49th general meeting, Soc. Am. Bact. p. 74.
13. Miller, J. M., Favour, C. B., Wilson, B. A. and Umbarger, M. A. 1949, A plasma factor responsible for *in vitro* lysis of leucocytes by tuberculoprotein, Proc. Soc. Exper. Biol. & Med. **70**: 738.
14. Miller, J. M., Favour, C. B., Wilson, B. A. and Umbarger, M. A. 1949, Nature of the plasma factor responsible for *in vitro* lysis of leucocytes by tuberculoprotein. Proc. Soc. Exper. Biol. & Med. **71**: 287.

OBSERVATIONS ON THE PRE-ERYTHROCYTIC STAGES OF *PLASMODIUM RELICTUM*, *PLASMODIUM CATHEMERIUM*, AND *PLASMODIUM GALLINACEUM* IN VARIOUS BIRDS

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During the course of our earlier efforts to elucidate the nature of that portion of the life cycle of avian malarial parasites occurring before patent parasitemia and later during various experiments involving studies of the pre-erythrocytic stages of these parasites, numerous observations were made on the pre-erythrocytic stages of *Plasmodium relictum*, *Plasmodium cathemerium* and *Plasmodium gallinaceum* which remain unpublished. These observations include (1) success or failure to observe pre-erythrocytic stages in a variety of avian hosts and (2) details of morphology and development of these stages hitherto unrecorded. Some generalizations follow these observations.

Huff and Coulston (1946) found that in addition to producing pre-erythrocytic stages in chickens *P. gallinaceum* could go through this early development in ducks and geese even though no parasitemia was produced in these two hosts. Likewise, *P. relictum* produced pre-erythrocytic stages in canaries and domestic pigeons even though in the latter parasitemia seldom developed. Huff (1948) tested the susceptibility of 14 species of Columbidae to *P. relictum* and found that pre-erythrocytic stages developed in only two of them (*Streptopelia senegalensis* and *Streptopelia humilis*). In the following paper 15 additional host-parasite combinations are reported.

MATERIALS AND METHODS

Strains 1P1-1 and 1W of *P. relictum*, strain 3Q of *P. cathemerium* and strain 8A of *P. gallinaceum* were used. *Culex pipiens* was used as the source of sporozoites for *P. cathemerium* and *P. relictum* while *Aedes aegypti* was used for *P. gallinaceum*. Details of the methods for transferring the infections by blood and by sporozoites, of preparing and examining the tissues for pre-erythrocytic stages and of the terminology used will be found in earlier publications (Huff and Coulston, 1944 and 1946) and are not repeated here. A standard inoculum of the salivary glands from 30 infected mosquitoes was used in the intracutaneous inoculation. In searching for the pre-erythrocytic stages in the skin, all sections containing remains of the inoculum and several sections on either side of the inoculum were thoroughly examined under the oil immersion lens. If difficulty was encountered in finding portions of the inoculum in the sections, the results were considered inconclusive, and in most such instances the results are not reported here. A subjective estimate of the degree of conclusiveness was entered in the notes at the time of examining the sections.

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The work here reported extends over the period 1941–1950 and was done in the Department of Bacteriology and Parasitology at the University of Chicago and at the Naval Medical Research Institute. A portion done at Chicago was under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Chicago. Another portion was supported in part by research grants from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service. During the period of the contract with the Office of Scientific Research and Development, Dr. Frederick Coulston collaborated with me.

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EXPERIMENTAL

1. New host-parasite combinations

A brief summary of new findings relative to the susceptibility of various birds to the pre-erythrocytic and erythrocytic stages of the three parasites following inoculation of sporozoites into the skin, together with previously reported findings, are incorporated into table 1. More detailed description of the new findings follows:

TABLE 1.—*Parasitism of tissues and blood of various birds inoculated with sporozoites into skin.*

Host Species	*P. relictum*		*P. cathemerium*		*P. gallinaceum*	
	Tissue findings	Parasitemia	Tissue findings	Parasitemia	Tissue findings	Parasitemia
Chicken (*Gallus gallus*)	−	−			++*	+++*
Guinea fowl (*Numida meleagris*)	−	−			−	−‖
Turkey (*Meleagris gallopavo*)	Normal cryptozoites	−			Normal cryptozoites	−
Pheasant (*Phasianus colchicus*)	Normal cryptozoites	−			Normal cryptozoites	+
California Valley quail (*Lophortyx californica*)					−	−
Duck (*Anas boschas domestica*)	Normal and abnormal cryptozoites	−	Normal cryptozoites	−	Normal and abnormal cryptozoites†	−†
Goose (*Anser anser domesticus*)					Normal cryptozoites†	−†
Pigeon (*Columbia livia*)	Normal cryptozoites†	−, +			−	+
Ring neck dove (*Streptopelia risoria*)	Negative§	−§	−	−	−	−
Canary (*Serinus canarius*)	Normal cryptozoites†‡	++†‡	Normal cryptozoites	++	Normal cryptozoites	−

* Huff and Coulston, 1944.
† Huff and Coulston, 1946.
‡ Coulston and Huff, 1947.
§ Huff, 1948.
‖ Two of 10 chickens subinoculated developed parasitemia.

P. relictum.—In the turkey normal appearing segmenters were found in sections of skin taken 67 hours after sporozoite inoculation. These were of the macro-type, characteristic of cryptozoites. However, in view of earlier findings (Coulston and Huff, 1947), 67 hours appears to be very late for finding segmenters from the first generation, and it is possible that the segmenters seen were of the second generation.

In half-grown ring pheasants (*Phasianus colchicus*) normal schizonts and segmenters were found 41 hours after injection of sporozoites. No differences were observed between these parasites and those described from the canary (Huff et al, 1947). No pre-erythrocytic forms were found in the skin of animals 72 hours after sporozoite inoculation. Inasmuch as the sections contained much debris from the inoculum and a thorough search was made in the vicinity of it, the negative findings appear to be fairly conclusive proof that the infection was overcome at the end of the first generation of parasites.

In the domestic duck, many cryptozoic segmenters and trophozoites of metacryptozoites were found 42 hours after sporozoite inoculation. Some of the segmenters (mature schizonts) were compactly filled with small, pink merozoites. A large proportion of the second generation trophozoites was abnormal in appearance. The partitions between

vacuoles were crooked and the vacuoles were abnormally large and irregular. One schizont and one segmenter had much the appearance of the forms of *Plasmodium lophurae* in the turkey which were interpreted as being abnormal (Huff et al, 1947).

Previously, it was reported (Huff and Coulston, 1946) that no parasitemia resulted from inoculation of sporozoites of strain 1Pl-1 of *P. relictum* into domestic pigeons, even though pre-erythrocytic stages were found in the local site of inoculation. Further experiments have shown that, while this is the rule, in some exceptions a slight parasitemia will result. Whether this difference in the susceptibility is an innate characteristic of the individual pigeon or is the reflection of a varying physiological state of the bird has served as the subject of further investigation.

The negative tissue findings in the ring-neck dove were obtained upon material fixed 40 and 70 hours after inoculation. These findings were considered, however, to be rather inconclusive on the basis of the histological study.

P. cathemerium.—Pre-erythrocytic stages of *P. cathemerium* were found in the local inoculation site of domestic ducks 42 hours after the inoculation was made. They were few in number and consisted of segmenters. One segmenter contained 26 loosely arranged merozoites with the usual vacuolate nuclei characteristic of other species fixed and stained by the same methods. However, one host cell containing a segmenter had a pycnotic nucleus. The merozoites were atypical in this parasite in that they contained central dark areas. It seems likely that both the host cell and its enclosed parasite were dead at the time of fixation. No parasites were found in ducks 72 hours after inoculation of sporozoites. As in the case of pheasants inoculated with sporozoites of *P. relictum* noted above, it would appear that the pre-erythrocytic infection was overcome by the host at the end of the first generation of parasites.

Abundant pre-erythrocytic stages of *P. cathemerium* were observed in the skin of canaries fixed 18 and 49 hours after inoculation. Since our observations on this species were made known only through progress reports to the Office of Scientific Research and Development (1943) and have not been published, the observations will be given somewhat more detailed description than the findings from the other species.

The parasites observed at 49 hours consisted of mature segmenting forms and second generation trophozoites. The morphology and staining of these parasites was so nearly like that already recorded for *P. relictum* (Coulston and Huff, 1947) that illustrations are not needed. It will suffice to point out the slight differences observed. In one segmenting form, bodies too large to be merozoites but not large enough or in the correct position to be considered as the central cores described for *P. gallinaceum* and *P. relictum* (Huff and Coulston, 1944; Coulston and Huff, 1947) were observed among the normal-sized merozoites. Similar structures were described from *P. lophurae* in turkeys (Huff et al, 1947, see fig. 10). However, in the latter there were also central aggregations of compactly arranged merozoites of smaller size and with deeper red stain which were lacking in the cathemerium segmenters. The average number of merozoites per segmenter was 35, which is somewhat larger than the average number for *P. relictum*. However, due to the difficulties of counting the merozoites in the segmenter and to the possibility that the section did not contain the entire parasite, this

may not be a valid difference between the two species. As with *P. relictum*, a certain proportion of both merozoites and trophozoites exhibited a lavender rather than azure coloration. The significance of this is not known although the possibility that the lavender (or pink) color may indicate death of the parasites has been mentioned previously (Coulston and Huff, 1947). In a few of the segmenters the merozoites were arranged entirely peripherally in the parasite, with no evidence of any kind of core or other central structure. There was much evidence of aggregation of the merozoites liberated from the segmenters into the adjacent tissues. As many as 13 trophozoites were found in one fibroblast. It is interesting to note that the metacryptozoic trophozoites at 49 hours were almost exactly the size of cryptozoites of 18 hours from another bird. This would indicate either (1) that segmentation began at about 31 hours or (2) that there is a considerable lag in the development of the sporozoites into cryptozoites. The latter possibility is somewhat discounted in *P. gallinaceum* by histological findings (Huff and Coulston, 1944).

At 18 hours after the inoculation of sporozoites, many trophozoites were observed in the macrophages of the skin. These were very similar to those of *P. relictum* and were mostly binucleate. The distribution of the growing cryptozoites in relation to the injected material was particularly apparent in the sections of this particular animal. The inoculum lay in a discrete mass. It consisted of the chitinous remains of the salivary glands and their attached tracheae, acidophil granulocytes, macrophages and lymphocytes. There was much karyorrhexis and disintegration of cells. It seems significant that not a single parasite was found in this mass although it must have contained the greatest concentration of sporozoites. On the other hand, parasites were numerous in macrophages immediately surrounding the area, some of them within a distance of one cell diameter from the injected mass. Since the granulocytes are the first cells to invade such an area, it is likely that they phagocytose and kill the majority of the sporozoites, and that the only sporozoites which survive are those that get quickly away from the area of intense invasion by granulocytes.

No pre-erythrocytic stages of *P. cathemerium* were found in ring-neck doves 42 hours after the inoculation of sporozoites. Unlike the observations on *P. relictum* in this host described above, the histological findings were fairly conclusive in this case.

P. gallinaceum.—Considerable additional material from chickens, especially from animals which received large inoculums of sporozoites, was carefully studied. These findings, which supplement our earlier papers (Huff and Coulston, 1944 and 1946), are given in a separate section below.

Negative but inconclusive results were obtained in one guinea fowl biopsied 72 hours after the inoculation of sporozoites. Two of 10 chickens subinoculated from the guinea fowl 5 days after sporozoite inoculation became infected.

Normal segmenting cryptozoites of *P. gallinaceum* were seen in the skin of a turkey biopsied 42 hours after the inoculation of sporozoites. Slight parasitemia was observed in the turkey.

In ring pheasants no pre-erythrocytic stages were seen in 5 animals biopsied at 40, 41, 46, 46, and 72 hours, respectively, after inoculation of sporozoites. In all except one of these, the results were fairly conclusive. Parasitemia appeared in one pheasant which had received two separate inoculums 16 days

after inoculation. The infection lasted seven days and did not attain a height of more than 3 parasites per 130 microscopic fields. Subinoculation was successful in one of two chickens; the infected one died with 75% of the erythrocytes infected.

Failure to find pre-erythrocytic stages of *P. gallinaceum* in canaries was reported earlier (Huff and Coulston, 1946). However, further study of the sections of canary skin biopsied 36 hours after the inoculation of sporozoites revealed a total of 5 segmenters, one of which appeared to be unusually small. Considering the amount of debris of mosquito origin in the sections, the number of parasites was remarkably small. Microscopic examination of the blood of the canaries and subinoculations to chickens failed to reveal any evidence of parasitemia. No pre-erythrocytic stages were found 70 hours after sporozoite inoculation.

In the domestic pigeon no pre-erythrocytic stages were found in skins biopsied at 42 and 70 hours after inoculation of sporozoites of *P. gallinaceum*. Two erythrocytic parasites were found in the pigeon on the 18th day and none on subsequent days. Parasitemia of very low grade resulted in one of four subinoculated chicks.

No pre-erythrocytic stages were found 41 hours after the injection of sporozoites into both ring-neck doves and California valley quail. There were also negative findings in the latter 72 hours after inoculation. No parasitemia resulted in either of these hosts, and no infections were established in chickens inoculated from either of them.

In an earlier study by Huff and Coulston (1946) pre-erythrocytic stages of *P. gallinaceum* were reported in geese and ducks, hosts which show a high degree of natural immunity to the erythrocytic stages. These were reported to be similar in every respect to the corresponding stages seen in chickens. A restudy of this material has revealed the presence of some abnormal pre-erythrocytic schizonts in ducks both at 48 and at 75 hours after inoculation of sporozoites. The abnormalities were similar to those described above for *P. relictum* in ducks and to those described (Huff et al, 1947) for *P. lophurae* in ducks, turkeys, chickens and guinea fowl.

2. *Supplemental notes on* P. gallinaceum *in chickens*

Localization.—During the early part of the prepatent period of infections resulting from the bites of a few mosquitoes or from the inoculation of the salivary glands from small numbers of infected mosquitoes, it was unusual to find any parasites microscopically in the tissues; and in the case of the former when an occasional one was found, it was usually seen in the spleen. It was hoped, therefore, that a more careful study of the tissues from those chicks which had received large intravenous inoculations of sporozoites would reveal some additional information on the localization of cryptozoites and metacryptozoites. Table 2 is a list of the tissues studied for this purpose.

Even though the study of these tissues has been more thorough and painstaking than was possible in our earlier study (1944), the results are almost exactly the same. The only cryptozoites seen in chicks inoculated intravenously with sporozoites were in the spleen. In spite of the fact that inoculations of the salivary glands from 200 and 300 infected mosquitoes were used, no parasites could be found in any of the organs of birds killed at 24 and 26 hours. After the first schizogony, however, parasites were found in all organs examined except the bone marrow. Con-

siderable differences were observed, however, in the distribution of parasites in individual birds; for example, in chick 2915 parasites were observed in the heart, spleen, liver, kidney and lamina propria, while in chick 5144 parasites were found in the brain and spleen but not in the heart, lung, liver, intestine, pancreas or kidney. The fact that the latter bird received a larger inoculum and was killed at a later time than the former would indicate even more clearly that the distribution of the parasites varies significantly from bird to bird. On a subjective basis, it appeared that the later generations of parasites were most abundant in the spleen and heart.

TABLE 2.—*Data on chicks which received large intravenous inoculations of sporozoites of* P. gallinaceum.

Bird no.	Number of mosquitoes used	Age of infection in hours	Microscopic findings (exoerythrocytic stages)
1474	300	24	Heart, lung, spleen, liver, negative
1462	200	26	All tissues negative
1480	200	36	Schizonts in spleen only
1458	200	48	Parasite in spleen only
1464	200	60	Parasites in heart, spleen, liver, brain and muscle
1439	170	70	Parasites in heart, spleen, liver, brain and kidneys
1478	200	72	Schizonts in liver, spleen, brain and kidney
2915	200	72	Parasites in heart, spleen, liver, kidney and lamina propria
5144	300	89	Parasites in spleen and brain. Heart, lung, liver, intestine, pancreas and kidney, negative
1459	100	90	All tissues positive except liver and bone marrow
2960	200	96	Parasites in heart, spleen, liver, brain and eye muscle
1457	200	120	All organs positive
685	200	120	Parasites in heart, spleen, liver and kidney Intestine, skin, thymus, bone marrow, pancreas, lung, gizzard, negative

Notes on morphology.—Additional study of the morphology of many thousands of pre-erythrocytic stages has confirmed all of the statements previously made by Huff and Coulston (1944) on this subject. In addition, some new findings resulted. In the brain capillaries of one bird (1457) longitudinal clefts were observed in the elongated schizonts. Shorter, transverse clefts were prevalent in nearly all large schizonts not only of this species but in all of others previously studied. However, the longitudinal clefts have not been previously observed except in *Leucocytozoon simondi* (Huff, 1942). The cleft depicted in figure 52 (plate 3) of the *Leucocytozoon* work is typical of those observed in the gallinaceum schizonts. Another type of cleft observed in the schizonts in the same bird was horseshoe shaped and again resembled those described in *L. simondi* (Huff, 1942, fig. 49, plate 3).

Schizonts in the pre-segmenter stage were observed to contain many small clefts. In some of them there was a suggestion that these clefts may be associated with some type of cytomery The clefts appear to become smaller and more numerous as the final production of merozoites is approached. A fairly large proportion of the mature schizonts (segmenters) studied contained merozoites and also the cleft-like divisions so common in younger schizonts. The combination of these characteristics in the same schizont gave the appearance of complete cytomeres. The structure of these schizonts should also be compared with similar stages in *Leucocytozoon* (Huff, 1942, see figs. 33, 34, 42, 43 and 54).

A careful restudy of sections of biopsied material from chickens previously immunized by sporozoite-induced infections revealed the presence of pre-erythrocytic stages of abnormal appearance, as well as of stages with normal appearance. The abnormalities were similar to the more extreme abnormalities described for *P. lophurae* in ducks (Huff, et al, 1947, see fig. 9), for *P. relictum* in ducks described above and for *P. gallinaceum* in chickens treated with sulfadiazine, napthoqui-

none and other drugs (Coulston and Huff, 1948).

DISCUSSION

Instances have previously been reported (Huff and Coulston, 1946; Coulston and Huff, 1947) in which sporozoites produced pre-erythrocytic infections in the sites into which they were inoculated, whereas no parasitemia developed in the same animals. To the three parasite-host combinations previously reported, the experiments described above add six more similar combinations, representing all three species of parasites and four hosts. The greater susceptibility of these hosts to pre-erythrocytic than to erythrocytic stages is also shown by the fact that out of the 21 parsite-host combinations in table 1, pre-erythrocytic stages were found in 13 (or 61.9%) of them, whereas erythrocytic infections were produced in only 6 (or 28.6%) of them. Taking into consideration the greater difficulty of finding parasites in the tissues than in the blood, it is probable that the actual susceptibility of these hosts to the pre-erythrocytic stages is even greater than the experimental results demonstrate. In fact, in some instances in which it has first appeared that hosts were completely insusceptible to the sporozoites of a species (i.e., canary vs. *P. gallinaceum*, and domestic pigeon vs. *P. relictum*), it has been shown in later observations that some pre-erythrocytic stages could be found in the tissues.

Even with the larger number of parasite-host combinations it is difficult to see any clear-cut relationship between the ability of sporozoites to develop in the tissues of hosts and the degree of consanguinity of the hosts in question. Normal cryptozoites of all three species of *Plasmodium* were observed in ducks and canaries and of *P. relictum* and *P. gallinaceum* in turkeys and pheasants. Of the 12 examples of pre-erythrocytic stages found 4 were from Galliformes, 4 from Anseriformes, 3 from Passeriformes, and one from Columbiformes. Furthermore, among the 9 parasite combinations with Galliform hosts there were 4 negative to both tissue and blood findings, 3 in which there were pre-erythrocytic stages but with no visible parasitemia and 2 in which both pre-erythrocytic and erythrocytic stages were found. It is also noteworthy that the susceptibility of the chicken to *P. gallinaceum* was, perhaps, one of the highest degree known, while the California valley quail, another gallinaceous bird, was not susceptible at all to the same parasite.

The association of degenerating (or abnormal appearing) pre-erythrocytic stages in the same host with normal stages has been previously noted in *P. lophurae* in chickens, ducks, turkeys and guinea fowl (Huff et al, 1947). A similar association was noted in the tissues of ducks infected either with *P. relictum* or *P. gallinaceum*. Similar abnormal forms of *P. gallinaceum* were seen in chickens immunized by previous sporozoite-induced infections. The cytological changes observed in each instance closely resemble those produced by certain antimalarial drugs (Coulston and Huff, 1948). It would, therefore, appear that regardless of whether the deleterious action is produced by the mechanisms of natural immunity, of acquired immunity or of drug effect, the degenerative changes in the pre-erythrocytic stages are similar.

The degree of similarity between the pre-erythrocytic stages of *P. cathemerium* and *P. relictum* is about what one should expect from the close similarity which exists between their erythrocytic stages. One observation on the

pre-erythrocytic stages of the former poses an interesting question. There was much evidence in the tissues that merozoites from the first generation localized in host cells closely adjacent to the segmenting parasite. Often these host cells were multiply infected, yet in no instance has there ever been evidence that merozoites start to grow into trophozoites while still in the original host cell. What prevents the merozoites from growing before the disruption of the host cell containing the segmenter? Inasmuch as there appears often to be ample room in the original host cell for at least the beginning of the development of the merozoites, it would appear probable that the metabolic products in and surrounding the merozoites must be the deterring influence.

The establishment of subpatent infections of *P. gallinaceum* in ducks and of *P. relictum* in domestic pigeons by the inoculation of sporozoites of these two species, respectively, has been previously reported (Huff and Coulston, 1946; Huff, 1947). Similar results were obtained in 4 species of pigeons and doves inoculated with sporozoites of *P. relictum* and in which there was no parasitemia microscopically visible (Huff, 1948). The establishment of such a subpatent infection of *P. gallinaceum* is here reported for the guinea fowl. Whether such subpatent infections are erythrocytic or exoerythrocytic in character is as yet unknown. It is interesting in either case to realize that subpatent infections of various species of malarial parasites can exist in hosts usually considered to be nonsusceptible to these parasites for months, perhaps even for years.

Failure to observe pre-erythrocytic stages microscopically for the first 24 hours after inoculation in the organs of chicks which had received intravenous inoculations of large numbers of sporozoites emphasizes again the extreme scarcity of the cryptozoic generation of parasites. Actual infection of kidney, muscle, spleen, pancreas and lung during the first 24 hours after sporozoite inoculation was proved previously by subinoculation experiments (Coulston et al, 1945). The development of sporozoites into cryptozoites has been observed in a connected series in the skin (Huff and Coulston, 1944). Therefore, failure to find cryptozoites microscopically in birds following intravenous inoculations should be attributed to their very great scarcity rather than to their complete absence from these organs. The microscopic findings after the first 36 hours (i.e., after the first schizogony) reported here, as well as those previously reported (Huff and Coulston, 1944), conclusively prove that second generation pre-erythrocytic stages may occur in nearly all organs. One notable exception is the bone marrow; all attempts to find parasites microscopically met with failure during the second generation. This was true even in birds in which parasites were plentiful in most of the other organs.

If experiments designed especially to reveal the early pre-erythrocytic stages have failed, it is not to be expected that one would meet with success in searching for the pre-erythrocytic stages in animals infected naturally or by smaller numbers of mosquito bites experimentally. It should also be borne in mind that in searching for the pre-erythrocytic stages of malarial parasites in animals larger than chicks the dilution of parasites in the host is also greatly increased by the size of the animal. For example, the inoculum for a man weighing 150 pounds would need to be 1360 times as large as that for a 50 g chick to assure a concentration in the tissues of the man comparable to that in the chick. Since intravenous inoc-

ulations of the sporozoites from 200 to 300 mosquitoes were used on chicks of that weight, we should need to use from 270,000 to 300,000 infected mosquitoes in order to obtain approximately the same concentration of parasites in the tissues of the man. It would, therefore, appear that the only hope of studying the early pre-erythrocytic stages of malarial parasites of large animals or man would probably lie in the use of techniques which would limit a large portion of the inoculum to localized areas.

In the present unsatisfactory state of knowledge concerning the classification of malarial parasites, any bit of information bearing on this subject is welcome. A theory of the evolution of malarial parasites based on the parallelism existing between the three genera of malarial parasites (*Plasmodium*, *Leucocytozoon* and *Haemoproteus*) and their respective invertebrate hosts was put forth earlier (Huff, 1938). As pointed out in 1942, the chief defect in this theory is that the genera *Haemoproteus* and *Leucocytozoon* are usually placed in one family (Haemoproteidae), whereas their respective vectors belong to cyclorrhaphous and orthorrhaphous diptera. The observations on the morphology of the exoerythrocytic stages of *P. gallinaecum* at least point toward a closer zoological relationship between *Plasmodium* and *Leucocytozoon* than is indicated by their present separation into separate families. If we admit the closer relationships between these two genera, the theory of the dipterous origin of malarial parasites is more acceptable, since the vectors of the two genera belong to the same suborder (Orthorrhapha) of the Diptera.

SUMMARY

1. Search for pre-erythrocytic stages of *Plasmodium relictum*, *Plasmodium cathemerium* and *Plasmodium gallinaceum* in 15 new parasite-host combinations resulted as follows:

a. Neither pre-erythrocytic nor erythrocytic stages were found in the following combinations: *P. relictum* in chickens or guinea fowl; *P. cathemerium* in ring-neck doves; *P. gallinaceum* in guinea fowl, California Valley quail or ring-neck doves.

b. Pre-erythrocytic but no erythrocytic stages were found in *P. relictum* in turkeys, pheasants and ducks; *P. cathemerium* in ducks; *P. gallinaceum* in turkeys and canaries.

c. Both pre-erythrocytic and erythrocytic stages were found in canaries inoculated with sporozoites of *P. cathemerium* and in pheasants inoculated with sporozoites of *P. gallinaceum*.

d. Erythrocytic but no pre-erythrocytic stages were found in domestic pigeons inoculated with sporozoites of *P. gallinaceum*.

2. In the 21 parasite-host combinations represented in this and previous work on these species of parasites, pre-erythrocytic stages were found in 13 (or 61.9%) of them, while erythrocytic stages were found in only 6 (or 28.6%) of them.

3. No clear relationship was discovered between the ability of sporozoites to develop in the tissues of hosts and the systematic classification of the hosts.

4. Degenerating pre-erythrocytic stages were observed along with normal ones in the tissues of ducks inoculated with sporozoites of either *P. relictum* or *P. gallinaceum*.

5. Degenerative changes in the pre-erythrocytic stages of *P. gallinaceum* in chickens immunized by previous infection were similar in appearance to the changes observed in corresponding stages effected either by natural immunity or by antimalarial drugs.

6. The morphology of the pre-erythrocytic stages of *P. cathemerium* was found to be very similar to that of *P. relictum*, but slight differences were observed.

7. An intensive search of the tissues of chicks which received intravenous inoculations of the sporozoites from 100 to 300 mosquitoes infected with *P. gallinaceum* revealed the presence of first generation (cryptozoic) stages only in the spleen. After the first schizogony all organs except bone marrow were found to be infected with pre-erythrocytic stages.

8. Morphological similarities between the exoerythrocytic stages of *P. gallinaceum* and of *Leucocytozoon* indicate a possibly closer systematic relationship between *Plasmodium* and *Leucocytozoon* than is generally believed.

REFERENCES

Coulston, F., Cantrell, W. and Huff, C. G. 1945, The distribution and localization of sporozoites and pre-erythrocytic stages in infections with *Plasmodium gallinaceum*. J. Infect. Dis. **76**: 226–238.

Coulston, F. and Huff, C. G. 1947, The morphology of cryptozoites and metacryptozoites of *Plasmodium relictum* and the relationship of these stages to parasitemia in canaries and pigeons. J. Infect. Dis. **80**: 209–217.

Coulston, F. and Huff, C. G. 1948, Symposium on exoerythrocytic forms of malarial parasites. IV. The chemotherapy and immunology of pre-erythrocytic stages in avian malaria. J. Parasitol. **34**: 290–299.

Huff, C. G. 1938, Studies on the evolution of some disease-producing organisms. Quart. Rev. Biol. **13**: 196–206.

Huff, C. G. 1942, Schizogony and gametocyte development in *Leucocytozoon simondi*, and comparisons with *Plasmodium* and *Haemoproteus*. J. Infect. Dis. **71**: 18–32.

Huff, C. G. 1948, Natural immunity and susceptibility of doves and pigeons to exoerythrocytic and erythrocytic stages of *Plasmodium relictum*. Proc. 4th Intern. Cong. Trop. Med. & Mal. **1**: 602–606.

Huff, C. G. and Coulston, F. 1943, Bi-monthly report no. 12, Comm. Med. Res. of the Office of Scientific Research & Development, June 1, 1943.

Huff, C. G. and Coulston, F. 1944, The development of *Plasmodium gallinaceum* from sporozoite to erythrocytic trophozoite. J. Infect. Dis. **75**: 231–249.

Huff, C. G. and Coulston, F. 1946, The relation of natural and acquired immunity of various avian hosts to the cryptozoites and metacryptozoites of *Plasmodium gallinaceum* and *Plasmodium relictum*. J. Infect Dis. **78**: 99–117.

Huff, C. G., Coulston, F., Laird, R. L. and Porter, R. J. 1947, Pre-erythrocytic development of *Plasmodium lophurae* in various hosts. J. Infect Dis. **81**: 7–13.

THE COURSE OF PNEUMOCOCCAL INFECTION IN MICE DURING TREATMENT WITH ANTIBACTERIAL SUBSTANCES AND ADRENOCORTICAL EXTRACT

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Interest in the part played by the adrenal cortex in resistance to infection, comprehensively reviewed by Perla,[1] has been revived by the successful use of adrenal steroids in rheumatic arthritis[2] and related disorders. Early attempts to alter the course of active infection by means of adrenal extracts generally were inconclusive, but in some instances beneficial effects were noted.[3] More recently it has been reported that treatment with adrenocortical extract prolonged the lives of guinea pigs infected with gas gangrene bacteria.[4,5] On the other hand, this extract given in conjunction with penicillin did not affect advanced pneumococcal infection in mice.[6]

In a previous report[7] it was shown that mice infected with pneumococci had improved chances of surviving if treated with adrenocortical extract, provided that the number of bacteria injected were small. The experiment was difficult to repeat, so that of four experiments only the first[7] and the fourth (table 1) gave significant results. Moreover, the use of highly diluted inoculums

TABLE 1.—*Survival of mice infected with light inoculums of type I pneumococci and treated with (7) 0.5 ml injections of adrenocortical extract or control solutions, without additional therapy.*

Time after infection (days)	Number of survivors									
	Dilution of inoculum* (log)									
	−8.0		−8.3		−8.6		−8.9		Pooled	
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
1	10	10	10	10	10	10	10	10	40	40
2	2		7	4	10	5	10	7	29	16
3			6	2	9	4	7	5	22	11
4			5	2	8	1	5	3	18	6
5			5	1	8	0	5	1	18	2
6			3	1	8		5		16	1
7			3		8		5		16	
8			3		8		5		16	
									($P = 0.001$)†	

* 0.5 ml intraperitoneal injection, serial half dilutions beginning with 10^{-8} undiluted culture approximately 1.2 billion diplococci per ml (Petroff-Hausser chamber count).

† Calculated according to a Chi-square formula (C. H. Goulden, 1929, Methods of Statistical Analysis, p. 106, John Wiley and Sons.

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1. Perla, D. and Marmorston, J. 1941, Natural Resistance and Clinical Medicine, Chap. 18, Boston, Little, Brown & Co.
2. Hench, P. S., Kendall, E. C., Slocumb, C. H. and Polley, H. F. 1949, Proc. Staff Meet., Mayo Clin. **24**: 181–197.
3. Pottenger, F. M. and Pottenger, J. E. 1937, Endocrinology, **21**: 529–535.
4. Kepl, M., Caldwell, G. and Ochsner, A. 1943, Proc. Soc. Exper. Biol. & Med. **52**: 25–26.
5. Vollmer, E. P., Cravitz, L. and Gillmore, J. D. 1947, Project X-759 Report No. 2, Naval Medical Research Inst., Bethesda.
6. Tobias, L., Jr. and Strauss, E. 1948, Proc. Soc. Exper. Biol. & Med. **69**: 529–531.
7. Vollmer, E. P. and Gillmore, J. D. 1947, Proj. X-759 Report No. 1, Naval Medical Research Inst., Bethesda.

of pneumococci left the experiment open to the criticism that some of the animals may have received blank injections. To obviate this it was decided to repeat the work in essentially the same form but with heavy inoculums of bacteria, held partially in check by chemotherapy. Such an experimental situation was of special interest because, as noted previously,[8] most if not all of the patients described as surviving the Waterhouse-Friderichsen syndrome between 1940 and 1947 had been treated both with hormones and antibacterial substances.

METHODS AND PROCEDURES

Swiss white mice, all males weighing 22 to 25 g, were used. Each mouse was injected intraperitoneally with 0.5 ml of 1 of 5 serial (10-fold) dilutions of a culture of virulent type I pneumococci. The numbers of diplococci in the undiluted cultures, as estimated by Petroff-Hausser chamber count, are given in context with their respective experiments. The dilutions used when the infection was to be weakened with a bacteriostatic agent were 10^{-3} to 10^{-7}, so that each mouse received 10 to 10,000 times as many pneumococci as the mice did in experiments without chemotherapy[7] (table 1). General bacteriologicl procedure has been described.[7] Random procedures of injection were followed to insure even dissemination of injection and equal handling of the mice. The blood of dead mice was checked for the presence of pneumococci by culture and slide smears up to the last day of each experiment.

In the first experiment with vitiated infection the mice were given 0.2% sulfadiazine mixed with ground food pellets. This diet, given two days before infection and thereafter for a week, had been found to retard the progress of infection and prolong the survival period without altering the terminal death rate. The culture used contained 0.4 billion diplococci per ml. Each hormone-treated animal received eight 0.5 ml injections of aqueous adrenocortical extract (ACE)* and four 0.1 ml injections of adrenal lipoextract† during the first 5 days of infection; controls received 0.5 ml of physiological saline solution when the aqueous extract was given.

In the second experiment two analogs‡ of pantothenic acid, *d*-pantoyltauryl-*p*-chloranalide and *d*-pantoyltauryl-3,4-dibromanalide, were used. A ground food meal containing 1% of each of these substances was found to retard the infection in preliminary trial, and since the supply of the drugs was limited, these proportions were used without further attempt to define their in vivo range of activity. All mice except 40 "no therapy" controls were put on this diet from 48 hours before to 48 hours after infection. The culture used contained approximately 2.0 billion diplococci per ml. Each of the hormone-treated mice was injected subcutaneously with 0.5 ml of ACE at 4, 22, 28, 33 and 46 hours after infection, and each control animal received 0.5 ml of physiological saline solution containing (as the ACE did) 10% ethyl alcohol.

RESULTS

For purposes of comparison, the results of an experiment involving treatment of infected mice with ACE but without chemotherapy have been presented (table 1). The survival of mice infected with light inoculums varied roughly with the differences in dilution of the inoculum, with the protective effect of the extract varying in like manner. A similar pattern was apparent when hormone-treated animals and their controls were injected with heavy inoculums of pneumococci and kept on diets containing antibacterial agents (tables 2 and 3). Thus, when sulfadiazine was used, the ACE-treated animals showed slight advantages throughout the dilution groups (table 2) and a significantly greater number of pooled survivors than that of controls during the first day that deaths occurred. At the end of the fourth day the difference between hormone-treated and control groups diminished, and the survival curves began to converge (fig. 1). Twenty "no therapy" controls were all dead before the end of the third day.

Similar results were obtained with the infection vitiated by means of the

8. Vollmer, E. P. 1949, Project NM 007 081.02.-08, Naval Medical Research Inst., Bethesda.

* Adrenal cortex extract (Upjohn) containing steroids biologically equivalent to 0.1 mg compound E. (Kendall) per ml.

† Lipoadrenal cortex (Upjohn), containing the equivalent of approximately 1.0 mg compound E per ml.

‡ Received from the American Cyanamide Co.

pantothenic acid analogs (table 3). The incubation period was shorter than in the previous experiment, but in the ensuing hours the hormone-treated animals for the most part resisted the infection better than the controls did, with a significantly greater number of survivors from the 34th to the 72nd hour ($P = 0.01$ to 0.05). The percentage of hormone-treated survivors exceeded that of controls until the end of the trial (fig. 2). Forty additional control animals receiving neither ACE nor antibacterial drugs were all dead within the second day of infection (fig. 2). Groups of ACE-treated animals appeared to be more active and more interested in water and food than were the comparable groups of controls.

TABLE 2.—*Survival of mice infected with heavy inoculums of type I pneumococci and treated with adrenocortical extract or control solution during therapy with 0.2% dietary sulfadiazine.*

Time after infection		Number of survivors												
		Dilution of inoculum (log)*												
		−3		−4		−5		−6		−7		Pooled		P
Day	Hour	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	
0	0	18	18	18	18	18	18	18	18	18	18	90	90	
3	72	16	14	18	17	18	17	18	16	18	18	88	82	0.10
	82†	15	9	17	12	15	14	18	15	18	17	83	67	<0.01
4	96	11	4	15	10	11	10	16	12	18	16	71	52	<0.01
	106	7	3	10	8	10	8	15	11	16	16	58	46	0.10
5	118	5	0	4	7	6	4	10	6	13	14	38	31	
	126	5	0	4	7	6	3	10	5	10	13	35	28	
6	142	4	0	3	4	4	2	6	3	7	7	24	16	
	150	4	0	1	4	4	2	5	3	6	4	20	13	
7		2	0	0	3	3	2	2	3	6	3	13	11	
8		2	0	0	3	0	0	2	2	3	2	7	7	

* 0.5 ml intraperitoneal injection, 10-fold serial dilutions from 10^{-4} to 10^{-7}; undiluted culture approximately 400 million diplococci per ml.
† The last 2 of 20 untreated controls (no sulfadiazine or hormone) died between the 72nd and 82nd hour. These had been injected only with the 10^{-7} dilution

TABLE 3.—*Survival of mice infected with heavy inoculums of type I pneumococci and treated with adrenocortical extract or control solution during therapy with pantothenate analogs.*

Time after infection		Number of survivors											
		Dilution of inoculum (log)*											
		−3		−4		−5		−6		−7		Combined	P
Day	Hour	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	
0	0	16	16	16	16	16	16	16	16	16	16	80	80	
1	24	16	15	16	16	16	16	16	16	16	16	80	79	
	27	16	12	16	16	16	16	16	16	16	16	80	76	
	30	14	12	16	14	16	15	16	16	16	16	78	73	
	34	13	7	16	11	16	15	16	16	15	16	76	65	0.02
2	48	7	4	11	5	15	14	14	15	15	10	62	48	0.05
	51†	6	3	10	4	14	12	14	13	15	10	59	42	0.01
	55	5	3	10	4	14	11	14	13	14	9	57	40	0.01
3	72	5	3	9	2	7	8	10	10	11	6	42	29	0.05
4		1	2	3	1	4	1	7	3	3	3	18	10	
5		1	2	2	0	3	1	6	3	3	3	15	9	
6		1	1	2	0	3	1	6	3	3	2	15	7	
7		1	0	2	0	3	1	6	3	3	2	15	6	
8		1	0	2	0	3	1	6	3	3	2	15	6	

* 0.5 ml intraperitoneal injection, 10-fold serial dilutions from 10^{-2} to 10^{-7}, undiluted culture approximately 2.0 billion diplococci per ml.
† The last of 40 untreated controls (no antipantothenate or hormone) died between the 48th and 51st hour.

DISCUSSION

These experiments show that lethal pneumococcal infection in mice passes through a phase when the administration of adrenal cortical extract prolongs survival time, without necessarily affecting the terminal survival rate. The experiment with sulfadiazine, in which the drug level was kept minimal, showed that this extended survival did not allow the mice to rid themselves of the pneu-

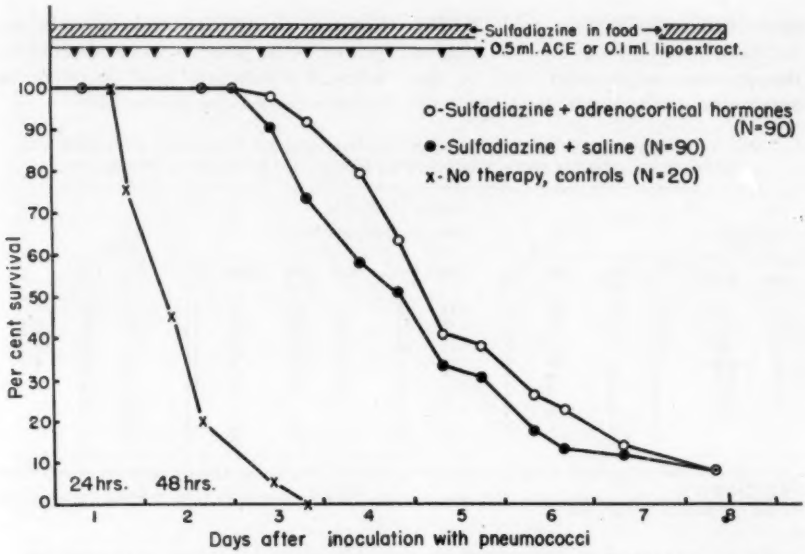

FIG. 1.—Survival curves of animals treated with sulfadiazine and adrenocortical hormones or saline solution.

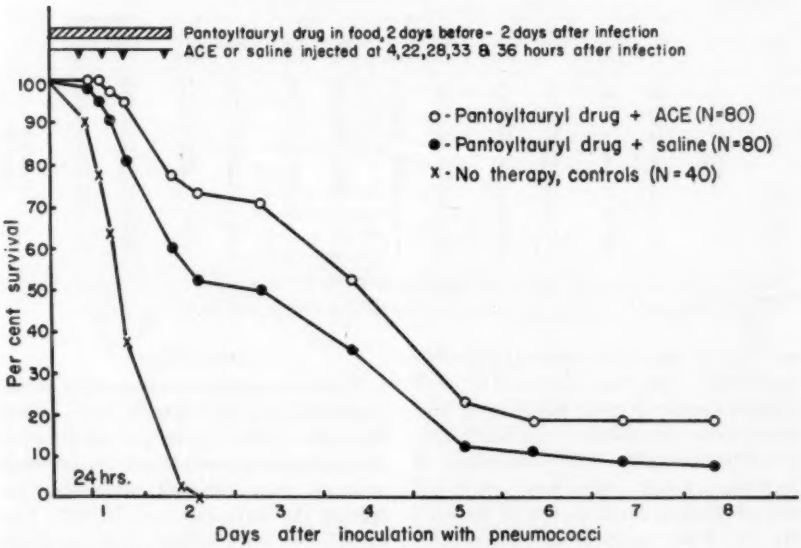
Pantoyltauryl drug in food, 2 days before - 2 days after infection
ACE or saline injected at 4, 22, 28, 33 & 36 hours after infection
o - Pantoyltauryl drug + ACE (N=80)
● - Pantoyltauryl drug + saline (N=80)
x - No therapy, controls (N=40)
Per cent survival
24 hrs.
Days after inoculation with pneumococci

FIG. 2.—Survival of infected mice treated with pantoyltauryl drugs and adrenocortical extract or saline solution.

mococci. The pantothenic acid analogs, however, were given in higher concentration, and although they were not given beyond the first two days of infection the additional advantage associated with the administration of the hormone appeared to be more sustained. It seems probable that further variation of the kinds and amounts of antibacterial substances used will bring out the adrenal cortical factor more clearly.

The adrenal cortex of mice infected with bacteria displays lowered lipid content and congestion of sinuses and capillaries,[9] and in other species bacterial vaccines or infections place the gland under a burden of extreme activity.[10–12] It is possible, therefore, that pneumococcal infection in mice leads to a relative adrenal insufficiency that shortens survival time and can be reversed by treatment with the hormone. The exact way in which adrenocortical function might affect resistance to infection is not clear, although the known physiology of the gland offers many possibilities for speculation (effects on protein and carbohydrate metabolism, distribution of electrolytes, etc.). Explanations depending upon specific mechanisms of defense in relation to adrenal function require more study. Thus, the existence of a simple stimulatory effect of adrenocortical hormone upon antibody formation[13] has not been supported.[14–16] The report[17] that desoxycorticosterone is bacteriostatic in vitro calls for confirmation; the effect described (about three times that of ethyl alcohol) does not appear great enough to assure in vivo activity. The extract used in the present work did not prevent growth of pneumococci in a concentration of 1 part in 16 in broth.[18] On the other hand, there have been convincing reports relating adrenocortical function with the phagocytic action of the macrophage system,[19] with resistance to toxins,[20,21] and with changes in tissue properties which might affect bacterial invasiveness.[22–25]

SUMMARY AND CONCLUSIONS

Swiss mice were infected intraperitoneally with large doses of type I pneumococci and kept on diets containing antibacterial substances to prolong the resulting infection. They were then injected with either adrenocortical extract or control saline solutions. In each of two experiments the survival time of hormone-treated animals was longer than that of controls, but the terminal survival rates were not significantly affected. It was concluded that the infected animals had suffered a relative insufficiency of adrenal cortical substances which was at least partially relieved by the administration of adrenocortical extract.

9. Deanesly, R. 1931, Am. J. Anat. **47**: 475–509.
10. Lithander, R. 1945, Acta. Med. Scand. (Suppl.) **160**: 1–114.
11. Pinchot, G. B., Close, V. P. and Long, C. N. H. 1949, Endocrinology, **45**: 135–142.
12. Vollmer, E. P. and Carey, M. M. 1949, Proj. NM 007 081.02.07 Naval Medical Research Inst., Bethesda.
13. Chase, J. H., White, A. and Dougherty, T. F. 1946, J. Immunol. **52**: 101–112.
14. Thatcher, J. S., Houghton, B. C. and Zeigler, C. H. 1948, Endocrinology, **43**: 440–447.
15. Herbert, P. A. and DeVries, J. A. 1949, Endocrinology, **44**: 259–273.
16. Vollmer, E. P. and Samsell, J. E. 1949, Endocrinology, **45**: 204–206.
17. Groeger, H. W. 1946, Arch. f. Hyg. **130**: 117–128.
18. Vollmer, E. P. Unpublished data.
19. Gordon, A. S. and Katsch, G. 1949, Ann. N. Y. Acad. Sci. **52**: 1–30.
20. Zwemer, R. L. and Jungeblut, C. W. 1935, Proc. Soc. Exper. Biol. & Med. **32**: 1583–1588.
21. Lewis, L. A. and Page, I. H. 1949, Ann. N. Y. Acad. Sc. **50**: 547–551.
22. Menkin, V. 1940, Am. J. Physiol. **129**: 691–697.
23. Baker, B. L., Ingle, D. J. and Evans, E. M. 1948, Anat. Rec. **102**: 313–332.
24. Ragan, C. 1949, Proc. Soc. Exper. Biol. & Med. **72**: 718–721.
25. Opsahl, L. J. 1949, Yale J. Biol. & Med. **21**: 255–262.

ST. LOUIS AND JAPANESE ENCEPHALITIS IN HAMSTERS FOLLOWING NATURAL FEEDING OF THE VIRUSES

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The susceptibility of the hamster (*Cricetus auratus*) to St. Louis encephalitis virus was reported in 1941 by Broun et al[1] shortly followed by a report by Lennette[2] that hamsters are also susceptible to Japanese encephalitis virus.

Broun et al[3] demonstrated circulating antibodies to St. Louis encephalitis virus in the blood of hamsters which had been fed the virus, and Mezera et al[4] reported that they had infected one hamster with the St. Louis virus after intraintestinal inoculation.

In 1944 Gordon[5] reported that when hamsters were allowed to feed naturally upon St. Louis encephalitis virus infected mouse brain, some developed clinical signs of infection and died, some developed clinical signs of infection and survived and the remainder, usually more than 50% of those fed the virus, developed no clinical signs of infection. He reported that all survivors of the feeding developed an immunity, as evidenced by the appearance of serum antibody or resistance to intracerebral challenge with many intracerebral lethal doses for normal hamsters of the homologous virus. He also found the same thing occurred when hamsters were allowed to feed naturally upon Japanese encephalitis virus infected mouse brain.[6]

The experiments reported here were threefold in purpose: first, to determine the distribution of Japanese and St. Louis encephalitis viruses in the tissues of hamsters after natural feeding in an attempt to determine the site or sites of multiplication of the viruses and possible pathways of spread following initial infection; second, to study the effects of age at time of feeding and the effect of thiamine deficiency on the relative numbers of clinical and subclinical infections in hamsters following natural feeding of the two viruses; and third, to further investigate the development of immunity in hamsters following feeding of the two viruses. Some of the results have been published previously in preliminary form[7,8]

MATERIALS AND METHODS

Viruses

The viruses used were the Hubbard strain of St. Louis encephalitis and the Nakayama strain

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1. Broun, G. O., Muether, R. O., Mezera, R. A. and Le Gier, M. 1941, Proc. Soc. Exper. Biol. & Med. **46**: 601–603.
2. Lennette, E. H. 1941, Proc. Soc. Exper. Biol. & Med. **47**: 178–181.
3. Broun, G. O., Le Gier, M., Mezera, R. A. and Muether, R. O. 1941, Proc. Soc. Exper. Biol. & Med. **48**: 310–313.
4. Mezera, R. A., Broun, G. O., Muether, R. O. and Le Gier, M. 1941, Proc. Soc. Exper. Biol. & Med. **48**: 357–359.
5. Gordon, F. B. 1944, J. Bact. **47**: 465 (abstract).
6. Gordon, F. B. Personal communication.
7. Schabel, F. M., Jr., Miller, S., Abendroth, M. and Gordon, F. B. 1947, Proc. Soc. Exper. Biol. & Med. **66**: 332–333.
8. Schabel, F. M., Jr., Gordon, F. B. and Abendroth, M. 1948, Proc. Meet. Soc. Am. Bact. **1**: 36 (abstract).

of Japanese encephalitis. These strains were originally obtained from Dr. Margaret Smith and had been carried through multiple intracerebral passages in mice in this laboratory. Stock pools of both viruses were prepared as follows: 75 white Swiss mice were inoculated intracerebrally with each virus. When signs of encephalitis were observed, the mice were sacrificed and their brains collected aseptically. Only those mice were used which were actually observed with symptoms. The mouse brains were weighed, emulsified by grinding in a mortar with sterile powdered glass and diluted to a final concentration of 10% by weight in veal infusion broth to which had been added 10% of sheep serum. These virus pools were centrifuged lightly and the supernatants dispensed in 2.0 ml amounts in glass tubes, stoppered with cotton and sealed with paraffin impregnated paper (parafilm). The virus pools were stored in a solid carbon dioxide chest with an observed temperature of about −65 C. These pools were used throughout the experiments reported here. After the pools had been in storage one week, samples of each were titrated in mice. Serial 10-fold dilutions were prepared, and 0.03 ml of each dilution from 10^{-6} through 10^{-9} was inoculated intracerebrally into each of 6 mice. The intracerebral LD_{50} of each virus for mice was calculated according to the method of Reed and Muench[9] and found to be $10^{-7.7}$ for the St. Louis virus and $10^{-7.5}$ for the Japanese virus.

Antiserums

Three rabbits were immunized to each virus. They were immunized as soon as they were received in the laboratory. Each weighed approximately 2 kg when received and increased about 200 g during the course of the immunization. Each animal was inoculated subcutaneously with 1.0 ml of one of the virus pools described above twice a week for six weeks. Ten days after the last inoculation each rabbit was exsanguinated, and the serums from the three rabbits inoculated with each virus were pooled and stored without preservative. Serums from four normal rabbits were pooled for use as a normal serum.

Identity of the viruses

Serum neutralization tests, using the stock viruses and antiserums to them, described above, indicated that the viruses were heterologous antigenically, and the slight but definite cross neutralization of the St. Louis virus by the antiserum to the Japanese virus is consistent with numerous other reports.[10] However, since the antiserums were prepared by inoculations of identical virus suspensions to which they were tested for neutralizing activity, the possibility of error in their identity was present. Samples of the antiserums described above were sent to the Department of Virus and Rickettsial Diseases of the Army Medical Department Research and Graduate School, Washington, D. C., for confirmation. Serum neutralization tests using the Hubbard strain of St. Louis encephalitis virus and the Nakayama strain of Japanese encephalitis virus in use there were in agreement with the results of the serum neutralization tests done in this laboratory and confirmed the identity of the viruses used.*

Animals

Hamsters.—The hamsters used were obtained from a single source.† The two- and three-week-old hamsters used were bred in the laboratory from breeding stock obtained from the same dealer.

Mice.—The mice used were Albino Swiss mice obtained from a single source.‡

Basic diet

The diet used to induce thiamine deficiencies in hamsters was a commercial preparation.¶ The manufacturer's analysis of the diet was as follows: sucrose, 68%, vitamin-free casein (hot alcohol extraction), 18%, vegetable oil, 10%, U.S.P. salt mixture no. 2, 4%.

Vitamin supplements.—Two supplementary vitamin preparations were used in the dietary experiments, one a mixture of water soluble vitamins and the second a mixture of the fat soluble vitamins.

Water soluble vitamin supplement.‖—All the water soluble vitamins used, except biotin, were

9. Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27**: 493–497.

10. Kudo, M., Uraguchi, K., Matsuda, S. and Hashimoto, H. 1937, J. Immunol. **32**: 129–135; Webster, L. T. 1940, Proc. Soc. Exper. Biol. & Med. **45**: 499–502; Smith, M. G. and Reames H. R. 1940, Proc. Soc. Exper. Biol. & Med. **45**: 838–842; Smithburn, K. C. 1942, J. Immunol. **44**: 25–31; Lennette, E. H., and Koprowski, H. 1946, J. Immunol. Virus Res. & Exper. Chemotherap., **52**: 235–246.

* The writer wishes to thank Dr. J. E. Smadel for conducting the neutralization tests.

† Haynor's Hamstery, Normal, Ill.

‡ Stanley Miller, Germantown, N. Y.

¶ G.B.I. vitamin B complex test diet, General Biochemicals, Inc., Chagrin Falls, Ohio.

‖ The writer wishes to thank Dr. James Moulder for help in preparing the water soluble vitamin mixtures.

crystalline preparations. They were suspended in the following concentrations in a 0.001 *M* monobasic and dibasic sodium phosphate buffer to which sodium ethylmercurithiosalicylate* to a final concentration of 1:1000 had been added as a preservative: riboflavin, 4 mg/ml, calcium pantothenate, 4 mg/ml, pyridoxine hydrochloride, 4 mg/ml, *p*-aminobenzoic acid, 8 mg/ml, choline chloride, 8 mg/ml, I-inositol, 8 mg/ml, nicotinic acid, 6 mg/ml, biotin,† 8μg/ml.

Thiamine hydrochloride to a concentration of 8 mg/ml was added to an aliquot sample of the above mixture for administration to control animals. The water soluble vitamin preparations were adjusted to a pH of 7.2 to 7.4 with sodium hydroxide and stored in brown bottles in a mechanical refrigerator.

Fat soluble vitamins.—A commercial preparation of the concentrated liver oils of percomorph fishes‡ was used as a source of vitamins A and D and as a carrier for vitamins E and K, which were added to it in the following concentrations: α-tocopherol (vitamin E)—50 mg/ml of oil, 2 methyl-naphthoquinone (vitamin K)—63 mg/ml of oil.

Feeding of the viruses to hamsters

At intervals when necessary, 40 or more mice were inoculated intracerebrally with one of the two viruses used. When the mice were observed with symptoms of encephalitis, they were sacrificed with diethyl ether and the heads skinned and removed. The lower jaw and tongue and attached muscle, and other tissues were removed, leaving essentially nothing more than the skull enclosing the brain. These mice heads were stored in the solid carbon dioxide ice chest until needed. Hamsters to be fed were placed in individual cages without food, water or litter, and held over-night. On the following morning, each hamster was offered one virus-infected mouse head which had been cut in two, exposing the brain. The animals were watched and when each one had eaten all the brain, it was returned to its regular cage. Hamsters consumed the mouse brains quickly, usually within one-half hour. Even thiamine-deficient hamsters, which had been showing extreme anorexia for some days, readily ate the mouse brains when offered. The only exception to the above procedure was the feeding of two-week-old hamsters. At this age they often died if held overnight in a bare cage. However, it was found that such hamsters would consume the mouse brain without a preliminary starvation so the food was not withheld before feeding of the virus to the two-week-old animals only.

In all of the experiments to be described, hamsters were held in individual cages.

EXPERIMENTAL

Distribution of St. Louis and Japanese encephalitis viruses in hamster tissues after natural feeding of the viruses

St. Louis encephalitis virus.—Each of a group of young adult hamsters was fed one St. Louis virus-infected mouse brain as described. At daily intervals for 6 days and each 2nd day from 6 to 12 days after feeding, 2 hamsters were sacrificed by exsanguination under light ether anaesthesia, and the tissues listed in table 1 tested for the presence of virus. Each tissue to be tested was carefully removed from the animal with a separate set of instruments. Tongue, esophagus, stomach and gut were repeatedly washed in sterile physiological saline and were thereby rendered sufficiently free of bacteria to allow intracerebral inoculation of mice. Each tissue was ground in a mortar with powdered glass and diluted in a minimal amount (from 0.5 ml for nasal mucosa and olfactory bulbs to 3.0 ml for cerebrum, depending on the size of the tissue) of serum broth. Each suspension was centrifuged lightly to sediment the ground glass and large tissue fragments, and the supernatant was inoculated intracerebrally into 3 or 4 mice. Blood

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* Merthiolate (Lilly).

† G.B.I. biotin concentrate no. 1000. General Biochemicals, Inc., Chagrin Falls, Ohio. This biotin preparation was in the form of a crude concentrate. The manufacturers state that it was prepared from a concentrated milk-sugar residue by adsorption and dilution methods, and that the concentration procedure probably removes all vitamins except biotin with the possible exception of trace amounts of nicotinic acid. The biotin preparation was not assayed for vitamin contaminants.

‡ Mead's Oleum Percomorphum.

specimens were taken by cardiac puncture and immediately injected intracerebrally into mice. Attempts to isolate virus from the stools were not made in this series of experiments because previous attempts were unsuccessful.[7]

Each hamster tissue tested (table 1) was considered to have contained the cause of illness or death, was passed to other mice by intracerebral inoculation using a simple technique described elsewhere.[11] This technique proved particularly useful in cases where the original inoculum was contaminated with bacteria.

Numerous second passage mouse brains were collected and the virus pres-

TABLE 1.—*Distribution of virus of St. Louis encephalitis in Hamsters after feeding upon it (3 experiments).*

Days after feeding	1	2	3	4	5	6	8	10	12
Buccal mucosa	00	00	00	00	0+*	0+	+0	00	00
Tongue	00	00	+0	+0	++	++	+0	00	00
Larynx	00	00	0+	00	++	0+	+0	0+	00
Cervical lymph nodes	+0	00	++	++	++	++	00	00	0+
Esophagus	00	00	00	00	0+	0+	+0	00	00
Stomach	00	00	00	00	00	00	00	00	00
Small gut	00	0+	0+	0+	++	+0	++	+0	0+
Lymphoid follicles of small gut	00	00	0+	0+	++	0+	0+	+0	0+
Large gut	00	00	0+	0+	0+	++	+0	00	00
Mesenteric lymph nodes	00	00	0+	++	++	++	+0	00	00
Inguinal lymph nodes	00	00	0+	++	++	00	00	00	00
Nasal mucosa	00	00	00	0+	0+	0+	+0	0+	00
Olfactory bulbs	00	0	0+	00	0+	00	+0	00	
Cerebrum	00	00	0+	00	+++	+	++0	00	
Brain stem	00	00	0+	00	++	00	+0	00	
Spinal cord	00	00	+0	00	++	00	+0	00	
Spleen	00	++	++	+0	++	00	+0	00	
Muscle	00	00	00	00	00	00	00	00	
Blood	000	++0	+++	++0	+++	000	000	000	000
	000	000	000	000	000	000	000	000	0
	000	000	000	000	00	0	0	0	
	0	0							

+, 0 = presence, absence of virus in tissue of one hamster as determined by inoculation of 3 or 4 mice.
* The viruses from 46 of the hamster tissues listed as (+) were carried through second intracerebral passages in mice and 12 of these second passage viruses were shown to be St. Louis encephalitis virus by serum neutralization tests with specific antiserum.

virus if the inoculated mice remained normal for three days after inoculation and then developed typical signs of St. Louis encephalitis infection (e.g., ruffling of fur, ataxia, hypersensitivity to touch or other mild stimuli, convulsions, ocular exudation) followed by death.

Numerous second intracerebral passages in mice were done from the first passage brain of those mice in which the above syndrome was observed. Second passages were always done if only one of the three or four mice inoculated with each specimen developed typical clinical signs of infection.

The brain of any mouse, seen ill or found dead following inoculation with a suspension of hamster tissue, and in which there was doubt concerning the ent identified as St. Louis encephalitis virus in serum neutralization tests with the rabbit antiserum described above.

The results indicate that the St. Louis virus became widespread in the hamster after feeding upon it, being recoverable from many tissues including the blood. The sequence of first appearance of virus in the various tissues tested does not necessarily indicate the sites of multiplication of the virus nor the path of spread through the body of the hamster. Viremia has been demonstrated as early as the 2nd day after feeding, and may occur at sometime in all hamsters, but its intermittent character indicates that the recovery of

11. Schabel, F. M., Jr. and Gordon, F. B. 1947, Science, **106**: 549.

virus from certain of the other extraneural tissues with a high degree of constancy from the 3rd to the 8th day after feeding (e.g., tongue, small intestine, lymphoid follicles of the small intestine, and mesenteric and cervical lymph nodes) is not due to their contained blood. Whether virus multiplies in these tissues, or accumulates there after multiplication at some other site cannot be determined from the data presented.

Japanese encephalitis virus.—Young adult hamsters were fed Japanese encephalitis virus-infected mouse brain and various tissues tested for the presence of the virus exactly as described for the St. Louis virus above. The results of these tests are listed in table 2.

TABLE 2.—*Distribution of virus of Japanese encephalitis in hamsters after feeding upon it (2 experiments).*

Days after feeding	1	2	3	4	5	6	8	10
Buccal mucosa	00	00	00	+0*	0+	00	00	0
Tongue	00	++	+0	++	++	+0	+0	0
Larynx	00	+0	++	++	00	00	+0	0
Cervical lymph nodes	+0	+0	+0	++	++	++	00	0
Esophagus	00	00	00	0+	0+	00	+0	0
Stomach	00	00	00	00	00	00	00	0
Small gut	00	0	00	0+	00	00	00	0
Lymphoid follicles of small gut	00	00	+0	++	+0	00	00	0
Large gut	00	+0	00	0+	0+	0+	00	0
Mesenteric lymph nodes	00	00	00	++	+0	0+	00	0
Inguinal lymph nodes	00	00	00	++	+0	++	00	0
Nasal mucosa	00	0+	+0	++	++	++	+0	0
Olfactory bulbs	00	00	+0	0+	++	++	++	0
Cerebrum	00	00	+0	0+	++	+0	++	0
Brain stem	00	00	00	++	++	00	++	0
Spinal cord	00	00	00	++	0+	+0	++	0
Blood	+++	+++	+++	+++	+0	000	000	000
	+0	+++	+++	+++	000	000	000	00
	00	++	++	+	0	0		

+, 0 = presence, absence of virus in tissue of one hamster as determined by inoculation of 3 or 4 mice.
* The viruses from 32 of the hamster tissues listed as (+) were carried through second intracerebral passages in mice and 12 of these second passage viruses were shown to be Japanese encephalitis virus by serum neutralization tests with specific antiserum.

The distribution of the Japanese virus in hamsters after feeding is similar to that of the St. Louis virus, with a few exceptions. Although found in the upper portion of the alimentary tract as constantly as was the St. Louis virus, it was less commonly found in the intestine and related lymphoid tissue. Viremia was present from the 1st through the 5th days after feeding, being present in all hamsters tested from the 2nd through the 4th days.

Recovery of St. Louis and Japanese encephalitis viruses from mouth swabs of infected hamsters

Clinical signs of infection develop in some hamsters following the feeding of mouse brain infected with either virus, characterized by one or more of the following symptoms: lethargy, ataxia, ocular exudation, wetting of the fur around the mouth, progressive paralysis of the extremities and, in some cases, death. In over 200 hamsters observed to develop clinical signs of infection following feeding of both viruses, the first clinically recognizable signs of infection appeared on the 5th to the 8th day after feeding.

Wetting of the fur around the mouth suggested that the ill animals may have had swallowing difficulty or hyperactivity of the salivary glands or both, possibly due to virus activity. Numerous attempts were made to isolate the viruses from the mouths of such animals. A small cotton swab was rubbed on the surface of the tongue, into the

buccal pouches and over the gums. The swab was washed out in 0.5 ml of 10% rabbit serum veal infusion broth containing 2000 units of crystalline penicillin G/ml,* centrifuged lightly, and the supernatant inoculated intracerebrally into 3 or 4 mice. By this method, both viruses were recovered from the mouth swabs of clinically ill hamsters showing wetting of the fur around the mouth, which had been infected by feeding the viruses; the St. Louis virus was recovered in 3 of 5 attempts on 3 hamsters, and the Japanese virus in 11 of 12 attempts on 12 hamsters (table 3).

To determine whether virus could be recovered from mouth swabs of virus-fed hamsters in the absence of clinical symptoms, the following experiment was done: Six young adult hamsters were fed Japanese virus-infected mouse brains as described above and mouth swabs tested at intervals up to 10 days after feeding. The virus was isolated 7 times on the 1st to 5th days after feeding, before any clinical signs of infection were apparent (table 4). Virus was isolated once from the one hamster, showing wetting around the mouth, on the 9th day after feeding. All of the virus isolations prior to onset of symptoms in the fed hamsters were made during the period of viremia (table 2) and all animals that had detectable virus in the mouth ultimately developed clinical symptoms of infection, although only one of them showed wetting around the mouth.

Since the virus isolations from mouth swabs reported above were all from animals which had been infected by

* The intracerebral infectious titer for mice of both viruses was not appreciably lowered by storage of a 10^{-3} dilution of infected mouse brain in the solid carbon dioxide ice chest for one week in the presence of 2000 units crystalline penicillin G/ml.

TABLE 3.—*Isolation of St. Louis and Japanese encephalitis viruses from mouths of hamsters infected by feeding.*

Hamster no.	Virus fed	Days after feeding of test	Result
71–18	St. Louis	6	+P,N
75–18	St. Louis	6	0
69–18	St. Louis	7	0
71–18	St. Louis	7	+P,N
75–18	St. Louis	7	+P,N
10–27	Japanese	6	+
30–27	Japanese	6	+
46–27	Japanese	6	+
48–27	Japanese	6	+
1–27	Japanese	7	+P,N
3–27	Japanese	7	+P,N
4–27	Japanese	7	+P,N
13–27	Japanese	7	+
20–27	Japanese	7	+
27–27	Japanese	7	0
37–27	Japanese	7	+
32–27	Japanese	8	+

+, 0 = presence or absence of virus as determined by inoculation of 3 or 4 mice.
P = virus carried through second intracerebral passage in mice for confirmation.
N = virus identified by serum neutralization tests.

TABLE 4.—*Relation of clinical signs to isolation of virus from mouth swabs of hamsters fed Japanese encephalitis virus.*

Hamster no.		Days after feeding								Remarks
		1	3	5	6	7	8	9	10	
21	Clinical signs	0	0	0	0	+	+	+	+	
	Salivating	0	0	0	0	0	+	+	+	Found dead p.m. 10th day
	Virus isolated	0	0	+	0	0	0	+		
22	Clinical signs	0	0	0	+					
	Salivating	0	0	0	0					Found dead 7th day
	Virus isolated	0	0	+	0					
23	Clinical signs	0	0	0	0	+	+	+	+	
	Salivating	0	0	0	0	0	0	0	0	Recovered, survived challenge
	Virus isolated	+	+	+	0	0	0		0	
24	Clinical signs	0	0	0	0	0	0	0	0	Survived challenge
	Salivating	0	0	0	0	0	0	0	0	
	Virus isolated	0	0	0	0	0	0	0	0	
25	Clinical signs	0	0	0	0	+	0	0	0	
	Salivating	0	0	0	0	0	0	0	0	Survived challenge
	Virus isolated	+	0	+	0	0	0	0	0	
26	Clinical signs	0	0	0	0	0	0	0	0	
	Salivating	0	0	0	0	0	0	0	0	Not challenged
	Virus isolated		0	0	0	0	0		0	

feeding, the possibility that small amounts of virus had persisted in the mouth as contamination from the feeding could not be ruled out, in spite of the fact that neither virus could be detected in mouth tissues (tables 1 and 2) 24 hours after feeding. To determine whether virus is present in the mouths of infected hamsters inoculated parenterally, mouth swabs of intracerebrally inoculated hamsters were tested. Hamsters were inoculated intracerebrally with about 100 LD_{50} of virus, and mouth swabs tested at intervals for the presence of virus. Both viruses were isolated from mouth swabs taken from intracerebrally inoculated hamsters, although, in the case of Japanese virus infections, with less frequency than from hamsters infected by feeding (table 5). It is of interest to note that none of the hamsters inoculated intracerebrally with either virus and from which mouth swabs were tested (table 5) showed wetting around the mouth. All animals tested died by the 6th day after inoculation, a considerably shorter period from inoculation to death than is generally observed in animals infected by feeding. The data indicate that virus can be present in the mouths of hamsters infected either by natural feeding or intracerebral inoculation of both viruses, but both viruses are recovered with the greatest frequency from the mouths of hamsters showing wetting around the mouth following infection by feeding. Whether the wetting around the mouth is due to impairment of the swallowing mechanism or stimulation of the salivary glands is not indicated by these studies. The excess salivation in rabies infections in animals has been attributed to hyperactivity of the salivary glands due to viral stimulation,[12] and a similar response to the Japanese and St. Louis viruses in hamsters infected by feeding may occur. Possibly the more rapidly fatal infection which follows the intracerebral inoculation of these viruses does not allow sufficient time for grossly obvious increased stimulation of the salivary glands.

TABLE 5.—*Isolation of Japanese and St. Louis encephalitis viruses from mouth swabs of intracerebrally inoculated hamsters.*

Days after inoculation	Japanese encephalitis				St. Louis encephalitis			
	Number of hamsters	Total tests	Virus recovered	Virus not recovered	Number of hamsters	Total tests	Virus recovered	Virus not recovered
1	9	9	1^{P}	8	3	3	0	3
2	9	9	$1^{P,N}$	8				
3	9	9	$2^{P,P}$	7	3	3	$2^{P,P}$	1
4	12	12	$1^{P,N}$	11	2	2	$1^{P,N}$	1
5	7	7	1^{P}	6	2	2	0	2
Totals	13	46	6	40	3	10	3	7

P = Virus carried through second intracerebral passage in mice for confirmation.
N = Virus identified by serum neutralization tests.

The possibility of virus containing blood being the source of virus in the mouth swabs tested is always present, but in the experiments reported here care was taken in collecting the swabs that no bleeding occurred. The swabs were examined for blood and none was seen, either on the cotton or in the suspending medium. However, in both virus infections in hamsters following feeding, the tongue was shown to contain virus (tables 1 and 2) during the time when the mouth swabs were positive. Large numbers of epithelial cells were present in the washings from the

12. Shortt, H. E. and Lahiri, B. N. 1934, Indian J. Med. Res. **21**: 587–604.

mouth swabs which were tested for virus, and these cells may have carried the virus from the infected underlying tissues from which they were being cast off.

Influence of age on susceptibility of the hamster to St. Louis and Japanese encephalitis viruses by feeding

There have been numerous reports that young animals are more susceptible to experimental virus infections than older ones. This age difference is most marked when animals susceptible to the so-called neurotropic viruses are inoculated by some parenteral route other than directly into the central nervous system (C.N.S.), adult animals usually showing the same susceptibility as younger animals to inoculation of these agents intracerebrally. This is true for herpes simplex,[13–15] Bwamba fever,[15] West Nile disease,[15] Japanese encephalitis,[15] St. Louis encephalitis,[15,16] pseudorabies,[14,15] equine encephalomyelitis,[15,17,18,19] spontaneous encephalomyelitis of mice,[20] poliomyelitis,[21,22] neurotropic yellow fever,[23] rabies,[24] and vesicular stomatitis.[25–27]

The influence of age on the susceptibility of experimental animals to infection with neurotropic viruses by feeding has been investigated much less extensively, infant rhesus monkeys having been infected experimentally by feeding Lansing poliomyelitis,[28] while the commonly used young rhesus monkey has been reported infected by feeding poliomyelitis virus only once.[29] Similarly, infant mice have been shown much more susceptible than adults to infection with murine SK poliomyelitis virus by gavage.[21]

Experiments were done to determine the influence of age on the susceptibility of hamsters to these viruses by feeding. Two, 3- and 6-week-old hamsters were fed virus-infected mouse brain as described. As a control, hamsters of the same ages were fed normal mouse brain. All animals were observed for 2 weeks, and survivors were challenged by inoculating them intracerebrally with multiple infectious doses of the same virus they were fed. (Intracerebral challenge doses in hamsters were from 10^5 to $10^{6.5}$ intracerebral LD_{50} for hamsters.) The results of these experiments are listed in table 6. Also included are a number of hamsters over 6 weeks of age, controls and otherwise from other experiments, in which circumstances allow their inclusion for comparative purposes.

It is evident that between 2- and 6-weeks-of-age hamsters acquire a definite resistance to clinically manifested encephalitis after feeding upon these

13. Andervont, H. B. 1929, J. Infect. Dis. **44**: 383–393.
14. King, L. 1940, J. Exper. Med. **72**: 573–593.
15. Lennette, E. H. and Koprowski, H. 1944, J. Immunol. Virus Res. & Exper. Chemotherap. **49**: 175–191.
16. O'Leary, J. L., Smith, M. G. and Reames, H. R. 1942, J. Exper. Med. **75**: 233–246.
17. Sabin, A. B. and Olitsky, P. K. 1938, Proc. Soc. Exper. Biol. & Med. **38**: 597–599.
18. King, L. 1940, J. Exper. Med. **71**: 95–106.
19. Morgan, I. M. 1941, J. Exper. Med. **74**: 115–132.
20. Theiler, M. 1937, J. Exper. Med. **65**: 705–719.
21. Jungeblut, C. W., Sanders, M. and Feiner, R. R. 1942, J. Exper. Med. **75**: 611–629.
22. Pinto, M. R. 1948, Am. J. Hyg. **48**: 361–380.
23. Bugher, J. C. 1941, Am. J. Trop. Med. **21**: 299–307.
24. Casals, J. 1940, J. Exper. Med. **72**: 445–451.
25. Sabin, A. B. and Olitsky, P. K. 1937, J. Exper. Med. **66**: 35–57.
26. Sabin, A. B. and Olitsky, P. K. 1938, J. Exper. Med. **67**: 229–249.
27. Sigurdson, B. 1943, J. Exper. Med. **78**: 17–26.
28. Horstmann, D. M., Melnick, J. L., Ward, R. and Sá Fleitas, J. 1947, J. Exper. Med. **86**: 309–323.
29. Melnick, J. L. and von Magnus, H. 1948, Am. J. Hyg. **48**: 107–112.

viruses. The data suggest that this resistance appears between the 2nd and 3rd weeks of life in the case of St. Louis encephalitis and after the 3rd week in the case of Japanese encephalitis. By the 6th week of life and beyond, 50% to 70% of hamsters show no clinical signs of illness after feeding upon either virus. However, the fact that all the animals which survived the feeding of virus resisted multiple intracerebral lethal doses and the fact that virus was detected in all fed animals from the 2nd through the 10th day after feeding St. Louis virus and from the 2nd through the 8th day after feeding Japanese virus (tables 1 and 2), indicate that all fed animals developed infection with these 2 viruses after feeding; and in those which did not succumb to the infection a solid immunity to intracerebral challenge with homologous virus resulted.

The results reported here suggest that age does not affect the susceptibility of hamsters to these viruses by feeding, since 50% to 70% of those 6 weeks of age and older, while showing no signs of infection, are probably infected after feeding (tables 1 and 2), and all survivors of feeding are resistant to many lethal doses of virus inoculated intracerebrally. Similar observations have been reported comparing the susceptibility of 7- and 21-day-old white rats to intranasal inoculation with St. Louis encephalitis virus.[30]

Effect of thiamine deficiency upon infection of hamsters following feeding of St. Louis and Japanese encephalitis viruses

The role of nutrition in experimental infections has been studied with a variety of hosts and infectious agents[31] and is known to influence virus infections in experimental animals. The obligate intracellular habitat of the viruses theoretically makes them especially influenced by factors which alter the normal metabolism of their particular chosen host cells, and indeed, in some experimental infections this has been shown to be true. Since thiamine deficiencies had been shown to decrease the susceptibility of mice to the Lansing strain of poliomyelitis virus,[32] as well

TABLE 6.—*Influence of age on susceptibility of hamsters to feeding with encephalitis viruses.*

Material fed	Age at feeding	Result			Challenge of survivors	
		Died with signs and/or virus in C.N.S.	Signs and Survived	No signs and survived	Challenge virus	Result
Japanese	2 wks	25	2	4 (13%)	Jaapnese	0/6*
Japanese	3 wks	22	5	4 (13%)	Japanese	0/9
Japanese	6 wks	8	0	22 (71%)	Japanese	0/22
Japanese	>6 wks	17	1	18 (50%)	Japanese	0/19
Normal brain	2 wks				Japanese	6/6
Normal brain	3 wks				Japanese	5/5
Normal brain	6 wks				Japanese	7/7
St. Louis	2 wks	26	3	1 (3%)	St. Louis	0/4
St. Louis	3 wks	12	5	13 (43%)	St. Louis	0/18
St. Louis	6 wks	11	3	16 (53%)	St. Louis	0/19
St. Louis	>6 wks	15	3	32 (64%)	St. Louis	0/35
Normal brain	2 wks				St. Louis	5/5
Normal brain	3 wks				St. Louis	5/5
Normal brain	6 wks				St. Louis	7/7

* Number dying/number inoculated.

30. Duffy, C. E. and Sabin, A. B. 1942, J. Bact. **43:** 88–89 (abstract).
31. Clark, P. F., McClung, L. F., Pinkerton, H., Price, W. H. and Schneider, H. A. 1949, Bact. Rev. **13:** 99–134.
32. Foster, C., Jones, J. H., Henle, W. and Dorfman, F. 1944, J. Exper. Med. **79:** 221–234; 1944, J. Exper. Med. **80:** 257–264; Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A. and Clark, P. F. 1944, J. Infect. Dis. **74:** 41–47.

as other neurotropic viruses,[31] its effect on susceptibility of hamsters to St. Louis and Japanese encephalitis viruses after feeding was tested.

The nutritional requirements of hamsters have been determined[32] and were considered in these experiments.

Several experiments were done as follows: Forty-two young hamsters (5 to 6 weeks old, weighing 40 to 55 g) were placed in individual cages on raised hardware cloth bottoms and separated into 3 groups of 14 animals each.

Group I. Thiamine deficient.—Each animal was given the basic diet and water ad libitum. Every 4th day each animal was weighed and given 0.5 ml of the mixture of water soluble vitamins without thiamine by gavage while under light ether anaesthesia. Each 8th day the water soluble vitamins were supplemented by 0.05 ml of the fat soluble vitamin mixture.

Group II. Partial inanition.—The animals in this group received water ad libitum, but the amount of the basic diet was limited. Each animal was fed only enough of the basic diet each day to maintain its body weight at approximately that of those animals being depleted in thiamine (group I). Each 4th day, each animal received the water soluble vitamins including thiamine, and each 8th day the fat soluble vitamins as described for group I.

Group III. Control. Complete diet.—The animals in this group received basic diet and water ad libitum, and each 4th day all the water soluble vitamins, and each 8th day the fat soluble vitamins as in group II.

Vitamin C is not necessary for the normal growth of hamsters[33] and was not included in the experimental diet.

The stools of hamsters on this diet are very scanty and readily passed through the hardware cloth bottom of the cages. Since the mesh bottom of the cage was one inch from the underlying pans, coprophagy was not likely in any case.

TABLE 7.—*Effect of thiamine deficiency and partial inanition on the susceptibility of hamsters to St. Louis and Japanese encephalitis viruses by feeding.*

Virus fed to hamster	Diet	Clinical signs and died	Clinical signs and survived	No clinical signs and survived	Challenge of survivors intracerebrally
St. Louis	Group I, thiamine deficient	8 (36%)		14 (64%)	0/12*
St. Louis	Group II, partial inanition	4 (40%)	1 (10%)	5 (50%)	0/6
St. Louis	Group III, complete diet control	3 (12%)	4 (16%)	18 (72%)	0/22
Japanese	Group I, thiamine deficient	8 (50%)	5 (31%)	3 (19%)	1/7
Japanese	Group II, partial inanition	7 (39%)	2 (11%)	9 (50%)	0/11
Japanese	Group III, complete diet control	14 (54%)		12 (46%)	0/12

* Number dying/number inoculated.

In the thiamine-deficient hamsters, the typical appearances of deficiency in the rat[34] were seen, characterized by emaciation, anorexia, lethargy, ruffling of the fur, and gastric erosions and ulcers.

All the hamsters were held on the regimen described for 16 days, at which time all were fed one mouse brain, infected either with St. Louis or Japanese encephalitis virus. The same dietary regimen was maintained for 14 days after feeding, at which time survivors were put on the normal laboratory diet and challenged intracerebrally with homologous virus. All animals dying

33. Cooperman, J. M., Waisman, H. A. and Elvehjem, C. A. 1943, Proc. Soc. Exper. Biol. & Med. **52**: 250–254; Hamilton, J. W. and Hogan, A. G. 1944, J. Nutrition, **27**: 213–224.

34. Eddy, W. H. and Dalldorf, G. 1944, The Avitaminoses, ed. 3, Baltimore, Williams and Wilkins Co.

during the observation period after feeding of virus were tested for the presence of virus in the brain by passage to three or four mice. Hamsters dying in the observation period from which no virus was isolated on passage were not included in the tabulation of results. Figure 1 shows typical weight curves for hamsters in the groups on different vitamin and caloric intakes.

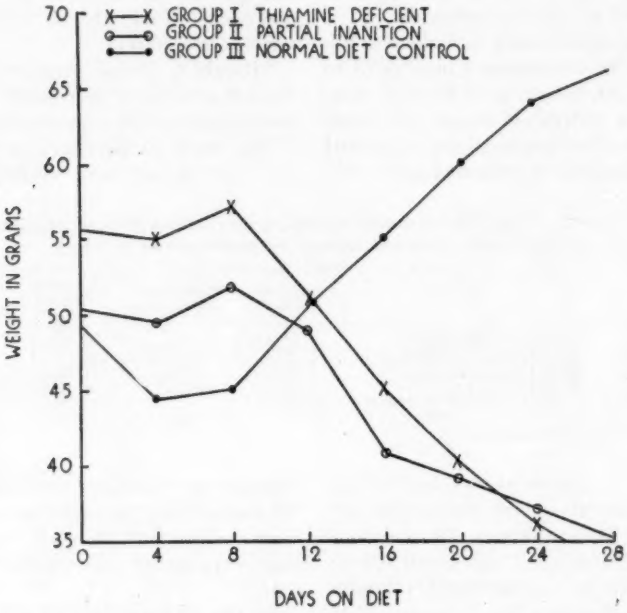

FIG. 1.—Typical weight curves for hamsters on thiamine deficient, low caloric and complete diets.

Two experiments using each virus were done and the results are shown in table 7. A comparison of the results in table 7 with those in table 6 indicates that thiamine deficiency or partial inanition does not appreciably affect the infection of hamsters with either virus following feeding. A somewhat lower percentage of hamsters deficient in thiamine and fed Japanese virus survived without the development of clinical signs of infection, but the percentage of survivors either with or without the development of clinical signs of infection is very similar in thiamine-deficient animals and in normal animals. The important finding is the observed resistance of all survivors (with one exception), irrespective of their nutritional state, to intracerebral challenge with homologous virus, indicating that thiamine deficiency or partial inanition in hamsters does not markedly alter their response to either virus by feeding.

Development of resistance to intracerebral inoculation with homologous virus after feeding St. Louis encephalitis virus to hamsters

Gordon[6] has found that following

feeding of these viruses to hamsters, resistance to intracerebral challenge with homologous virus develops in some of the fed animals in a very short time, in some cases in 3 to 4 days. Attempts were made to determine how quickly after feeding virus resistance to intracerebral challenge could be demonstrated.

Twelve young adult hamsters were each fed one St. Louis virus-infected mouse brain and 12 others were each fed one normal mouse brain. At daily intervals, two hamsters from each group were inoculated intracerebrally with $10^{5.2}$ intracerebral LD_{50} of St. Louis virus. The results are shown in table 8. It will be noted that on the 3rd, 4th and 6th days after feeding virus, one of the two animals tested resisted the intracerebral challenge that killed all the hamsters which had been fed normal mouse brain.

TABLE 8.—*Development in hamsters of resistance to intracerebral challenge with St. Louis encephalitis virus after feeding the virus.*

Interval from feeding to intracerebral challenge	Day of death after intracerebral challenge	
	Fed St. Louis virus	Fed normal mouse brain
1 day	4, 6	4, 6
2 days	5, 11	4, 10
3 days	5, survived	5, 5
4 days	4, survived	5, 6
5 days	7, 1	6, 6
6 days	13, survived	5, 6

In a second experiment, attempts were made to determine what relationship neutralizing antibodies might have to this rapidly appearing intracerebral resistance. A group of 36 hamsters were each fed one St. Louis virus-infected mouse brain. Each day after feeding all hamsters were bled by cardiac puncture, after which 2 to 4 of them were inoculated intracerebrally with $10^{5.2}$ intracerebral LD_{50} of St. Louis virus. After any given animal had been chal-

TABLE 9.—*Resistance of hamsters to intracerebral challenge with St. Louis encephalitis virus after feeding.*

Hamster no.	Days after feeding virus							Results of intracerebral challenge		
	1	2	3	4	5	6	7		Day of death after	
								Died or survived	Feeding	Intracerebral challenge
1	Bled									
	Inoc. i.c.*							Died	7	6
3	Inoc. i.c.							Died	7	6
4	Bled	Bled								
		Inoc. i.c.	Group I					Died	8	6
5	Bled	Inoc. i.c.						Died	7	5
6	Bled	Inoc. i.c.						Died	6	4
8	Bled	Bled								
			Bled							
			Inoc. i.c.					Died	7	4
9	Bled	Bled	Inoc. i.c.	Group II				Died	8	5
10	Bled	Bled	Inoc. i.c.					Died	8	5
11	Bled	Bled	Inoc. i.c.					Survived		
12	Bled	Bled	Bled							
				Bled						
				Inoc. i.c.				Survived		
13	Bled	Bled	Bled	Inoc. i.c.	Group III			Survived		
14	Bled	Bled	Bled	Inoc. i.c.				Survived		
15	Bled	Bled	Bled	Inoc. i.c.				Died	8	4
16	Bled	Bled	Bled	Bled						
					Bled					
					Inoc. i.c.			Survived		
17	Bled	Bled	Bled	Bled	Inoc. i.c.	Group IV		Survived		
18	Bled	Bled	Bled	Bled	Inoc. i.c.			Survived		
21	Bled	Bled	Bled	Bled	Inoc. i.c.			Survived		
26	Bled	Bled	Bled	Bled	Bled					
						Bled				
						Inoc. i.c.		Died	8	2
27	Bled	Bled	Bled	Bled	Bled	Inoc. i.c.		Died	8	2
30	Bled	Bled	Bled	Bled	Bled	Inoc. i.c.	Group V	Died	7	1
32	Bled	Bled	Bled	Bled	Bled	Inoc. i.c.		Died	8	2
33	Bled	Bled	Bled	Bled	Bled	Bled				
							Bled			
							Inoc. i.c.	Died	11	4
34	Bled	Bled	Bled	Bled	Bled	Bled	Inoc. i.c.	Died	13	6
36	Bled	Bled	Bled	Bled	Bled	Bled	Inoc. i.c.	Survived		

* Inoculated intracerebrally with St. Louis virus.

lenged with virus intracerebrally, daily bleedings were discontinued. By this method daily serum samples after feeding and up to the time of intracerebral challenge were available on a group of animals. Table 9 shows the various bleedings and results of intracerebral challenge. The table lists only 24 of the original 36 animals fed virus. The 12 missing are ones which died as a result of trauma from the repeated cardiac punctures or which developed signs of encephalitis from the virus exposure from feeding. No animal was challenged intracerebrally which was showing any clinical signs of infection at the time of challenge. By the 4th day after feeding the virus, most of the animals are resistant to challenge with multiple intracerebral lethal doses of virus. The animals in group V, table 9, were probably heavily infected prior to the intracerebral challenge and would have died of the virus infection even if the intracerebral challenge had not been done, since it will be noted that the interval from intracerebral challenge to death is short. In fact, we have not observed death from intracerebral inoculation in control hamsters before the 4th day after inoculation. In addition, the interval from feeding to death in the same group (group V, table 9) is typical of that seen in normal hamsters fed St. Louis virus but not challenged intracerebrally. Thus it appears that fatal infections induced by ingestion of the virus were developing in the animals in group V, table 9, and the intracerebral challenge did not appreciably alter the course of the infection in these animals.

To determine if the observed resistance to intracerebral challenge was due to humoral antibody, the serums of the hamsters in each group in table 8 were pooled by days and tested for neutralizing antibodies to the St. Louis virus. Virus dilutions were mixed with equal parts of the undiluted hamster serum pools so that the final virus concentrations were in serial 10-fold dilutions of infected mouse brain. The

TABLE 10.—*Serum neutralization test with pooled hamster serums taken at daily intervals after feeding and before intracerebral challenge with St. Louis encephalitis virus*

Group no.*	Number of hamsters in group	Result of challenge	Interval from feeding to challenge	Serum neutralization test	
				Day after feeding	Result
I	3	Died	2 days	1st	Negative
I	3	Died	2 days	2nd	Negative
II	3	Died	3 days	1st	Negative
II	3	Died	3 days	2nd	Negative
II	3	Died	3 days	3rd	Negative
III	3	Survived	4 days	1st	Negative
III	3	Survived	4 days	2nd	Negative
III	3	Survived	4 days	3rd	Negative
III	3	Survived	4 days	4th	Negative
IV	4	Survived	5 days	1st	Negative
IV	4	Survived	5 days	2nd	Negative
IV	4	Survived	5 days	3rd	Negative
IV	4	Survived	5 days	4th	Negative
IV	4	Survived	5 days	5th	Negative
V	4	Died	6 days	1st	Negative
V	4	Died	6 days	2nd	Negative
V	4	Died	6 days	3rd	Negative
V	4	Died	6 days	4th	Negative
V	4	Died	6 days	5th	Negative
V	4	Died	6 days	6th	Negativ
Pooled serums from 5 hamsters fed St. Louis virus infected mouse brain 4 times at 3 to 4 day intervals. Bled 15 days after last feeding					Positive
Pooled serums from 5 hamsters fed normal mouse brains 4 times at 3 to 4 day intervals. Bled 15 days after last feeding					Negative
Pooled serums from 10 unfed hamsters					Negative
Hyperimmune rabbit serum					Positive
Veal-infusion broth, plus 10% sheep serum control					Negative

* These are the same groups of hamsters as listed in table 9.

To determine if the observed resistance to intracerebral challenge was due to humoral antibody, the serums of the hamsters in each group in table 8 were pooled by days and tested for neutralizing antibodies to the St. Louis virus. Virus dilutions were mixed with equal parts of the undiluted hamster serum pools so that the final virus concentrations were in serial 10-fold dilutions of infected mouse brain. The

mixtures were incubated and inoculated intracerebrally into mice. A pool of normal hamster serum was used as a control. The results of the neutralization tests are listed in table 10. In no pool of hamster serum were neutralizing antibodies detected by this method. The technique used in the serum neutralization test does not indicate whether or not small amounts of antibody were present in the hamster serums, and it is not unlikely that some feeding (table 9) may all have had C.N.S. infections at the time of challenge, although direct evidence for this is lacking.

The rapid development of resistance to challenge with multiple lethal doses of St. Louis virus,[37,38] lymphocytic choriomeningitis virus[39] and eastern equine encephalomyelitis virus[40] has been reported. The fact that no detectable neutralizing antibodies were present at a time when resistance to challenge was

TABLE 11.—*Cross immunity between Japanese and St. Louis encephalitis viruses in hamsters.**

Intracerebral inoculum	Immune to Japanese encephalitis virus			Immune to St. Louis encephalitis virus		
	Died	Survived with clinical signs	Survived without clinical signs	Died	Survived with clinical signs	Survived without clinical signs
0.05 ml 10^{-1} St. Louis virus-infected mouse brain	4 (12%)	7 (20%)	23 (68%)	0	0	81 (100%)
0.05 ml 10^{-1} Japanese virus-infected mouse brain	1	0	67 (98%)	12 (44%)	4 (15%)	11 (41%)

* The hamsters challenged with homologous virus had not been previously inoculated intracerebrally, while those challenged with heterologous virus had previously resisted intracerebral challenge with homologous virus.

small amounts would have been detected if a more delicate test had been used.[35] However, the results indicate that sufficient antibody was not present to account for the observed resistance to intracerebral challenge in these animals.

The results suggest that this might be another example of active immunity to superinfection similar to the so-called "Magrassi phenomenon" in herpes febrilis infections in rabbits.[36] On the 4th and 5th day after feeding St. Louis virus, all hamsters tested were shown to harbor virus in some tissues, and 3 of the 5 tested had virus in the C.N.S. (table 1). It is not unlikely that those animals resistant to intracerebral challenge on the 4th and 5th days after high in the virus-fed hamsters reported here is similar to the findings reported in St. Louis encephalitis,[37,38,41] equine encephalomyelitis[41,42] and rabies[43] in mice.

Cross immunity between St. Louis and Japanese encephalitis viruses in hamsters

In the course of these studies, a number of hamsters became available which had been fed one or the other of the viruses and survived, either with or without having developed clinical signs

35. Olitsky, P. K. and Harford, C. G. 1938, J. Exper. Med. **68**: 173–189; Lennette, E. H. and Koprowski, H. 1944, J. Immunol. **49**: 375–385.
36. Magrassi, F. 1935, Ztschr. f. Hyg. u. Infektiouskr. **117**: 501–528.
37. Webster, L. T. 1938, J. Exper. Med. **68**: 111–124.
38. Hodes, H. L. and Webster, L. T. 1938, J. Exper. Med. **68**: 263–271.
39. Lyon, R. A. 1940, Pub. Health Rep. **55**: 2178–2180.
40. Morgan, I. M. 1941, J. Exper. Med. **74**: 115–132.
41. Casals, J. 1943, J. Exper. Med. **78**: 447–463.
42. Morgan, I. M. and Olitsky, P. K. 1941, J. Immunol. **42**: 445–454.
43. Casals, J. 1940, J. Exper. Med. **72**: 453–461.

of infection. All but one of these animals resisted intracerebral challenge with multiple lethal doses of homologous virus (table 11).

Two weeks after intracerebral challenge with homologous virus some of these animals were inoculated intracerebrally with heterologous virus. (The heterologous virus challenge doses were not titrated but were 10^4 or more lethal intracerebral doses for normal young adult hamsters.) The results of the cross immunity tests are in table 11. The results indicate that 68% of Japanese virus immune hamsters resisted challenge with St. Louis virus, and 41% of St. Louis virus immune hamsters resisted challenge with Japanese virus. Only 12% of Japanese virus immune animals died, but nearly one-third developed clinical signs with or without death following challenge with St. Louis virus; while 44% of St. Louis virus immune animals died and nearly two-thirds developed clinical signs with or without death following challenge with Japanese virus. This cross immunity indicates, in a general way, a relationship between these two viruses similar to that which has been shown by serum neutralization tests.[10] However, by serum neutralization tests, the degree of neutralization of St. Louis virus by antiserum to the Japanese virus is much less than the cross immunity studies here would indicate and generally very little, if any, neutralization of Japanese virus by antiserum to the St. Louis virus is observed. The experimental studies of neutralizing antibodies to and resistance to challenge with the St. Louis virus, at least in immune mice,[37,38,41] show no correlation between the two. The results of cross immunity studies reported here would indicate a closer antigenic relationship between the two viruses than do serum neutralization tests, if indeed, the cross immunity observed is specific. In considering the specificity of the observed cross immunity, the studies of Schlesinger, Olitsky and Morgan[44] must be considered. They showed that guinea pigs, rabbits and mice immunized to western equine encephalomyelitis virus and surviving intracerebral inoculation with the homologous virus were immune for about two weeks to intracerebral inoculation with multiple intracerebral lethal doses of eastern equine encephalomyelitis virus. Guinea pigs immune to and surviving challenge with western equine encephalomyelitis virus resisted the New Jersey strain of vesicular stomatitis virus injected intracerebrally, and mice in the incubation period or paralyzed following intracerebral inoculation with Theiler's virus showed an increased resistance to western equine encephalomyelitis virus inoculated intracerebrally.

The recent report of Bodian[45] has indicated a similar occurrence in monkeys infected with the virus of poliomyelitis. He reported that monkeys acutely ill with one serologic type of poliomyelitis virus are usually resistant to intracerebral inoculation with a heterologous strain, while convalescent monkeys are susceptible to intracerebral inoculation with a heterologous strain.

Although neither St. Louis or Japanese encephalitis viruses were isolated from the C.N.S. of hamsters more than 8 days after feeding (tables 1 and 2), the possibility that some residual nonspecific effect due to recent infection with either virus being responsible for the apparent cross immunity between these viruses as indicated by the cross challenge experiments (table 10) cannot be ruled out.

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44. Schlesinger, R. W., Olitsky, P. K. and Morgan, I. M. 1943, Proc. Soc. Exper. Biol. & Med. **54**: 272–273; 1944, J. Exper. Med. **80**: 197–211.

45. Bodian, D. 1949, Am. J. Hyg. **49**: 200–224.

The duration of resistance to challenge with heterologous virus in hamsters resistant to intracerebral inoculation with the same virus which had been fed was not extensively investigated. In a small group of animals (12) which had been fed St. Louis virus and resisted intracerebral challenge with homologous virus 2 weeks after feeding, all tested (6 of 6) resisted intracerebral challenge with homologous virus 3 months after feeding, while all tested (6 of 6) died following intracerebral challenge with the Japanese virus 3 months after feeding. These results are quite different from those found on cross challenge at a shorter interval after feeding either virus (table 11), but they do little to answer the question of whether or not the observed cross immunity (table 11) is specific or not since these viruses are known to be related immunologically.[10] The stimulus to the original immune state was the St. Louis virus, and it is quite possible that immunity to Japanese virus induced by the related St. Louis virus would not be as enduring as that to the homologous St. Louis virus.

DISCUSSION

The data presented do not unequivocally establish that these viruses multiply in non-nervous tissue of the hamster after ingestion of the virus, but the presence of virus in blood, spleen, superficial lymph nodes, tongue and larynx before their appearance in the C.N.S. and their appearance in a variety of tissues other than the C.N.S. simultaneously with their first appearance in the C.N.S. indicate that they probably do multiply, if not extraneurally, at least outside of the C.N.S.

The distribution of St. Louis encephalitis virus in mice following intravenous, intracerebral and intranasal inoculation has been studied by Peck and Sabin.[46] Their data did not indicate extensive virus multiplication outside the C.N.S. prior to multiplication of virus within it, but in the terminal stages of infection the virus was present in as high a concentration in some non-nervous tissue (e.g., leg muscle, heart, lungs, spleen, tongue and urinary bladder) as in the sciatic nerve. Since the amount of virus in the blood is negligible at that time, they concluded that the virus was accumulating in these tissues by diffusion from nerve fibers along which centrifugal spread from the C.N.S. is occurring, or that it was multiplying in some other constituent of these tissues. Similarly, in the data presented here (tables 1 and 2) it will be seen that both the Japanese and St. Louis encephalitis viruses are present in a variety of tissues other than the C.N.S. at a time when virus is no longer present in the blood.

Similar observations have been made on the widespread distribution of other neurotropic viruses in the viscera and other tissues of experimentally infected animals, e.g., lymphocytic choriomeningitis[47] and poliomyelitis,[28,48] and in naturally occurring poliomyelitis in man.[49]

The question of whether or not the so-called neurotropic viruses can multiply in non-nervous tissue has recently received considerable attention, and from several reports it appears that at least some of them e.g., Japanese encephalitis virus,[50] lymphocytic chorio-

46. Peck, J. L. and Sabin, A. B. 1947, J. Exper. Med. **85**: 647–662.
47. Armstrong, C., Wooley, J. G. and Onstott, R. H. 1936, U. S. Pub. Health Rep. **51**: 298–303.
48. Sabin, A. B. 1944, J. Mt. Sinai Hosp. **11**: 185–206.
49. Sabin, A. B. and Ward, R. 1941, J. Exper. Med. **73**: 771–793.
50. Gordon, F. B., Schabel, F. M., Jr. and Abendroth, M. 1948, Federation Proc. **7**: 271.

meningitis virus,[51] Semliki Forest virus[51] and poliomyelitis virus[52–54] do multiply, if not extraneurally, at least outside the C.N.S. Intramuscular inoculation of some neurotropic viruses, e.g., St. Louis encephalitis virus[55] and GD VII or FA strains of mouse encephalomyelitis viruses,[56] cause extensive necrotic lesions of muscle fibers which are, at least in the latter case, neutralized by specific antiserum.[56]

Many of the unsolved problems concerning the epidemiology of numerous virus diseases of man and animals will probably be clarified with the demonstration of inapparent infections in at present unknown reservoirs. Inapparent infections are known to occur in a number of virus diseases, and at least in some, e.g., poliomyelitis[57] and lymphocytic choriomeningitis,[58] the majority of infections in the most common natural host are inapparent. The virus of eastern equine encephalomyelitis was not known to be present in the Philippine Islands until the appearance of a spontaneously infected native monkey[59] prompted the examination of horse and chicken serums for neutralizing antibodies to this virus. These studies indicated that inapparent infections in horses had probably occurred.[60]

On the basis of serum neutralization tests it appears likely that inapparent infections with Japanese encephalitis virus occur in several domestic animals in Japan where epizootics have not been recognized[61] as well as in humans in areas of China where epidemics have not been known to occur.[62]

While the weight of evidence indicates arthropod transmission as the most frequent and most likely means of dissemination for many of the viral encephalitides, a very considerable number of them are known to be transmissible either experimentally or in nature or both, by ingestion of the various viruses, e.g., mouse-hamster virus,[63] St. Louis encephalitis virus,[64] lymphocytic choriomeningitis virus,[65] Ilhéus encephalitis virus,[66] pseudorabies virus[67] and murine SK poliomyelitis virus.[68]

The experiments reported here suggest that the viruses of St. Louis and Japanese encephalitis may be carried in

51 Overman, J. R. and Friedewald, W. F. 1950, J. Exper. Med. **91**: 39–51.

52 Evans, C. A. and Green, R. G. 1947, J.A.M.A. **134**: 1154–1155.

53. Enders, J. F., Weller, T. H. and Robbins, F. C. 1949, Science, **109**: 85–87.

54. Weller, T. H., Robbins, F. C. and Enders, J. F. 1949, Proc. Soc. Exper. Biol. & Med. **72**: 153–155.

55. Sabin, A. B. and Ward, R. Unpublished data cited in Peck and Sabin.[19]

56. Rustigian, R. and Pappenheimer, A. M. 1949, J. Exper. Med. **89**: 69–92.

57. Casey, A. E., Fishbein, W. I., Schabel, F. M., Jr., Smith, H. T. 1950, Am. J. Pub. Health, **40**: 1241–1250.

58. van Rooyen, C. E. and Rhodes, A. J. 1948, Virus Diseases of Man, ed. 2, New York, Thomas Nelson and Sons.

59. Livesay, H. R. 1949, J. Infect. Dis. **84**: 306–309.

60. Mace, D. L., Ott, R. L. and Cortez, F. S. 1949, Bull. U. S. Army Med. Dept. **9**: 504–507.

61. Sabin, A. B., Ginder, D. R. and Matumoto, M. 1947, Am. J. Hyg. **46**: 341–355.

62. Sabin, A. B., Schlesinger, R. W. and Ginder, D. R. 1947, Proc. Soc. Exper. Biol. & Med. **65**: 183–187.

63. Bolin, V. and Anderson, J. A. 1947, Proc. Soc. Exper. Biol. & Med. **66**: 518–522.

64. Harford, C. G., Sulkin, S. E. and Bronfenbrenner, J. 1939, Proc. Soc. Exper. Biol. & Med. **41**: 332–333.

65. Milzer, A. 1942, J. Infect. Dis. **70**: 152–172.

66. Koprowski, H. and Hughes, T. P. 1946, J. Immunol. Virus Res. and Exper. Chemotherap., **54**: 371–385.

67. Remlinger, P. and Bailly, J. 1934, Ann. Inst. Pasteur (Paris), **52**: 361–405; Shope, R. 1935, J. Exper. Med. **62**: 85–89, 101–117.

68. Jungeblut, C. W., Sanders, M. and Feiner, R. R. 1942, J. Exper. Med. **75**: 611–629; Schultz, E. W. and White, S. C. 1948, Proc. Soc. Exper. Biol. & Med. **68**: 266–274.

some unknown animal reservoir following ingestion of the viruses, as well as other means of transmission. Data presented here indicate that these viruses do not long persist in hamsters after they have fed upon them, but the St. Louis virus has been recovered as long as 162 days after intranasal inoculation of passively immune mice,[69] and some unknown reservoir host to either virus might well harbor virus for long periods after infection acquired by natural feeding.

SUMMARY

Following feeding of St. Louis encephalitis virus to hamsters (*Cricetus auratus*), the virus is recoverable from many tissues including the blood. The sequence of first appearance of virus in the various tissues does not indicate the sites of multiplication nor the path of spread in the body of the hamster. Viremia was demonstrated as early as the second day after feeding, and probably occurs at some time in all fed hamsters, but its intermittent character gives evidence that the recovery of virus from certain of the other extraneural tissues is not due to their contained blood. The data do not indicate whether or not the virus multiplies in these extraneural tissues or merely accumulates in them after multiplication in some other site.

The distribution of Japanese encephalitis virus in the tissues of the hamster after feeding upon the virus is similar to that seen with St. Louis virus, except that it is found less frequently in the intestine and related lymph tissues and more constantly in the blood.

Both St. Louis and Japanese encephalitis viruses were recovered from mouth swabs of hamsters infected with each virus, either by feeding or by intracerebral inoculation.

The ratio of hamsters developing clinical signs of infection to those with subclinical infection after feeding either virus is markedly affected by age. Following feeding of Japanese virus, 87% of 2- and 3-week-old hamsters developed clinical signs, while at 6 weeks and older only 40% developed clinical signs. Following feeding of St. Louis virus, 97% of 2-week-old hamsters, 57% of 3-week-old hamsters and 38% of 6-week and older hamsters developed clinical signs of infection. All hamsters which survived feeding of the virus, irrespective of age at time of feeding, or whether or not clinical signs of infection developed after feeding on either virus, resisted intracerebral challenge with homologous virus.

Thiamine deficiency or partial inanition does not affect the susceptibility of hamsters to either virus by feeding.

Resistance to intracerebral challenge with St. Louis encephalitis virus, not attributable to circulating neutralizing antibody, develops in some hamsters in four to five days after feeding on the virus.

Hamsters surviving feeding and subsequent intracerebral challenge with either virus show a marked resistance to intracerebral challenge with the heterologous virus.

A possible interpretation of the findings reported here in relation to the natural history of the viral encephalitides is discussed.

69. Slavin, H. B. 1943, J. Bact. **46**: 113–116.

IN VITRO AND CHEMOTHERAPEUTIC STUDIES WITH CHLORAMPHENICOL (CHLOROMYCETIN*) AGAINST *HEMOPHILUS PERTUSSIS*

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Although the reports of Hegarty et al (1945) and Brownlee and Bushby (1948) showed that experimental *Hemophilus pertussis* infection in mice did not respond well to treatment with streptomycin or certain of the sulfonamide drugs, the more favorable results with aerosporin by the latter authors and the report of McLean et al (1949) on in vitro studies with chloramphenicol suggested further in vitro tests and a chemotherapeutic trial with chloramphenicol.

IN VITRO TESTS

The in vitro effect of chloramphenicol on *H. pertussis* was tested by the blood agar plate dilution method. Plates of Bordet-Gengou agar base with human blood and containing various concentrations of chloramphenicol were surface-seeded with approximately 10,000 organisms of various recently isolated strains of Phase I *H. pertussis* and the virulent strain No. 18323 of Kendrick et al (1947) used in pertussis vaccine potency tests. Inhibition levels were read after 96 hours incubation at 35–36 C. Because it was deemed advisable to test the drug in the presence of serum, this plate method did not permit further testing for bacteriocidal effects.

Table I shows that the seven strains tested were inhibited by drug concentrations of 0.2 to 0.3 μg per ml of medium as compared with 2.6 μg of streptomycin base. Repeated testing of subcultures showed the effective concentration of drug required for complete inhibition of growth to vary but slightly.

TABLE 1.—*Chloramphenicol vs.* H. pertussis *in vitro.*

Strain	Growth in 96 hours					
	Chloramphenicol: μg/ml				Streptomycin (base) μg/ml	
	0.05	0.1	0.2	0.3	1.3	2.6
18323	+	+	+	–	+	–
04688	+	+	+	–	+	–
04722	+	+	P	–		
04734	+	+	P	–		
04723	+	+	–	–		
04732	+	P	–	–		
04743	+	P	–	–		

+ = full growth
P = partial growth
– = no growth

CHEMOTHERAPEUTIC TESTS

1. Intranasal infection

The chemotherapeutic activity of chloramphenicol was first determined against the pneumonic type of infection in mice produced by intranasal inoculation, because we felt that this type more nearly simulated the natural form of the disease. It was found early in our studies that when careful attention was given to the physiological age of the challenge culture, reproducible results in the LD_{50} inoculum and the subsequent multiple challenge inoculum could be assured. In our study we employed culture No. 18323 because of its known mouse virulence and because, of the 7 *H. pertussis* strains tested in vitro, it had the greatest resistance to chloramphenicol. Dried cultures were vitalized by 72 hour culture on Bordet-Gengou rabbit blood agar and subcultured at 48 hour intervals. Twenty-four hours before mice were to be infected the culture was seeded onto a flask of Bordet-Gengou agar containing no peptone, subsequently removed from the medium by scraping, suspended in 1% amigen solution and standardized by lumetron count. Only the third or fourth subcultures were used for challenge.

White mice weighing from 15 to 16 g were lightly etherized and given intranasally by droplet instillation an infectious dose of 1.25 billion organisms contained in 0.05 ml which was approximately 10 to 15 LD_{50} doses. Only those mice were included which apparently had inhaled the total dosage, eliminating those mice in which "bubbling" or oral regurgitation occurred. The virulence of the culture was checked by titration in the range of the LD_{50} level.

Treatment with chloramphenicol and sulfadiazine was initiated approximately 1 hour after infection and continued twice daily at the beginning and close of the laboratory day for 1, 2,

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* Parke, Davis & Co. trade name.

4, 8 and 12 day periods. Mice received drugs in 0.25 ml of 10% acacia solution orally by cannula and control groups received the acacia solution only. Mice dying within 24 hours of challenge were considered accidental deaths and were not included in the experiment. All surviving animals were held for 22 days, and mice dying during the course of the experiment were autopsied, the lungs examined macroscopically and cultured for *H. pertussis* on B-G slants.

In an effort to ascertain whether progressive infection was being stopped by bringing about the death of the infective organism, temporary inhibition of growth or by attenuation of the organism, surviving mice were sacrificed and careful examination for *H. pertussis* made in smears of lung tissue and by cultivation on Bordet-Gengou medium.

Determination of therapeutic dosage.—Table 2 shows the determination of optimal dosage of chloramphenicol and sulfadiazine. Chloramphenicol in dosages of 1.75 mg and 3.5 mg given twice daily gave equal protection (100%) and were superior to the 0.875 mg level (42%). Sulfadiazine showed only a slight superiority of the 10 mg over the 5 mg dosage (70% and 59%). From these findings, we chose for further experiments therapeutic dosages of 2 mg of chloramphenicol and 10 mg of sulfadiazine, both twice daily.

TABLE 2.—*Therapeutic dosage of chloramphenicol and sulfadiazine in mouse pertussis.*

Infecting dose	Drug	Amt. of Drug B.I.D. ×12	Survivals (22 days)	
1.25B	Chloramphenicol	0.875 mg	15/36	42%
"	"	1.75 "	36/36	100%
"	"	3.5 "	36/36	100%
"	Sulfadiazine	5.0 "	22/37	59%
"	"	10.0 "	25/37	70%
"	None		4/20	20%

Chemotherapeutic effect in varied treatment periods.—Tables 3 and 4 are protocols of typical experiments chosen to represent the two extremes of mortality in the control groups which were encountered in our series of experiments. In the chloramphenicol-treated groups survival rates were 95% in groups treated 1, 2 and 4 days, with 5% survival in the controls, as compared to 100% survival after 2 days chloramphenicol treatment when the control survival rate was 40%. Sulfadiazine protected 40%, 40% and 60% of mice in respective groups in the first experiment and 60% in the second experiment. It is evident therefore, that this variation

TABLE 3.—*Chemotherapy of* H. pertussis *in mice—Exp. 19.*

Infecting dose/0.05 ml I.N.	No. of mice	Oral treatment B.I.D.	Days of treatment	Day of death	Survivals (22 days)	Deaths	% Survivals
1.25 B	21	2 mg chloramphenicol	1	17	20	1	95
"	21	"	2	11	20	1	95
"	21	"	4	5	20	1	95
"	20	10 mg sulfadiazine	1	5, 8, 8, 9, 9, 11, 13, 14, 14, 17, 20, 20	8	12	40
	20	"	2	5, 8, 8, 9, 9, 11, 11, 12, 14, 15, 15, 16	8	12	40
"	20	"	4	5, 8, 9, 14, 20, 20, 20, 20	12	8	60
"	20	None	0	4, 4, 6, 8, 10, 11, 11, 13, 13, 15, 16, 16, 17, 19, 19, 20, 20, 20, 20	1	19	5
125 M	10	"	0	12, 13, 15, 15, 18	5	5	50

TABLE 4.—*Chemotherapy of* H. pertussis *in mice—Exp. 15.*

Infecting dose/0.05 ml I.N.	No. of mice	Oral treatment B.I.D.	Days of treatment	Day of death	Survivals (22 days)	Deaths	% Survivals
1.25 B	20	2 mg chloramphenicol	2		20	0	100
"	20	10 mg sulfadiazine	2	4, 5, 5, 5, 11, 17, 17 (5 mice sick)	12	8	60
"	20	None	0	4, 4, 4, 4, 7, 9, 9, 11, 12, 12, 13, 21 (3 mice sick)	8	12	40
125 M	10	"	0	12, 14, 21 (4 mice sick)	7	3	70

in the lethal effect of the challenge culture had no significant effect on the chemotherapeutic evaluation of the drugs.

Table 5 summarizes 6 experiments in which treatment was given for periods of 1 to 12 days. Mice receiving 2 mg chloramphenicol twice daily for periods of 1 and 12 days had survival rates of 95% to 100% as compared with survival rates of 40% to 60% for mice receiving 10 mg sulfadiazine twice daily on the same schedule and 20% survivals among the controls. From these results it would appear that there is little difference in the 1 or 12 day periods of treatment with chloramphenicol. However, in mice receiving sulfadiazine, the longer period of treatment showed an advantage over the shorter periods.

TABLE 5.—*Chloramphenicol chemotherapy of mouse pertussis* (summary).

Drug:	Chloramphenicol		Sulfadiazine		Control			
Challenge I.N.:	1.25 B		1.25 B		1.25 B		125 M	
Treatment:	2 mg B.I.D.		10 mg B.I.D.		None			
22 Days Surv.:	No.	%	No.	%	No.	%	No.	%
Days Treated								
0					19/95	20	23/50	46
1	20/21	95	8/20	40				
2	39/40	97.5	20/40	50				
4	59/61	97	27/60	45				
8	19/19	100	12/20	60				
12	17/17	100	12/20	60				

Recovery of H. pertussis *from surviving mice.*—Results of cultural studies on mice surviving the experiments included in Table 5 showed that *H. pertussis* was recovered from 90% of control mice, 50% of sulfadiazine-treated groups and 4% of chloramphenicol-treated mice. On the basis of these data, and in view of the fact that these examinations were made at periods of 10 to 21 days after cessation of drug treatment, we believe that chloramphenicol has exerted or brought about a lethal effect in the course of treatment and has not created a so-called "carrier-state."

2. *Intracerebral infection*

So that the chemotherapeutic effects of these drugs against the pneumonic type of infection could be compared with the chemotherapeutic effect of drugs on the classical intracerebral infections usually employed for tests with *H. pertussis*, the following experiments were designed.

White mice weighing 18 to 20 g were injected intracerebrally with 100,000 *H. pertussis* organisms of strain No. 18323 contained in 0.03 ml of 1% amigen solution. Control groups received 100,000, 2000, 400 and 80 organisms per 0.03 ml inoculum respectively. The same precautions as previously described were used to control the physiological age of the culture. Approximately one hour after infection, oral treatment with chloramphenicol and sulfadiazine was started, repeated in 5 hours and then continued twice daily for periods of 4 and 12 days using the same technique as described for the treatment of intranasally injected mice. Mice dying during the first 3 days of treatment were not included in the experiment. Mice were held for 21 days.

Tables 6 and 7 show the effect of 4 and 12 days of treatment with the two drugs. Again, as was shown in the intranasal experiments, there was no advantage of the longer over the shorter treatment periods. Sulfadiazine protected 50% of the mice in both of these experiments, which is in the protection range determined in intranasal experiments summarized in Table 5, and chloramphenicol protected only 27% and 40% of the mice in the 4 and 12 day treatment regimens. It is evident that both drugs apparently had some effect

on prolonging the survival time over that of the control group. The failure of chloramphenicol to protect mice infected intracerebrally in spite of increasing the daily dosage from 4 mg to 7 mg, is not surprising in view of the report of Glazko et al (1949) on the low concentration of chloramphenicol in the fluids and tissues of the central nervous system as compared with blood levels; also to be considered is the known ease of absorption of sulfadiazine into the central nervous system.

3. Chloramphenicol exerts a lethal effect on pertussis organisms causing pneumonic infection in mice as determined by available laboratory confirmatory tests.

TABLE 6.—*Chemotherapy of intracerebral pertussis.*

No. of mice	Infecting dose I.C.	Treatment B.I.D. ×4	Day of deaths	Survivals (22 days)	Deaths	% Survivals
22	100,000	3.5 mg chloramphenicol	9, 9, 10, 10, 11, 11, 11, 11, 12, 12, 12, 13, 13, 16, 20, 21	6	16	27
22	100,000	10 mg sulfadiazine	10, 11, 11, 12, 12, 13, 13, 13, 13, 18, 21	11	11	50
10	100,000	None	6, 6, 7, 7, 9, 9, 9, 13, 20, P*	0	10	0
10	2,000	"	6, 7, 9, 9, 11, 13, 13, 16, P	1	9	10
10	400	"	11, 11, 12, 13, 13	5	5	50
10	80	"	13	9	1	90

* Paralyzed on 21st day.

TABLE 7.—*Chemotherapy of intracerebral pertussis.*

No. of mice	Infecting dose I.C.	Treatment B.I.D. ×12	Day of deaths	Survivals	Deaths	% Survivals
20	100,000	3.5 mg chloramphenicol	16, 16, 16, 17, 17, 18, 18, 19, 19, 19, 21, P*	8	12	40
20	100,000	10 mg sulfadiazine	18, 18, 19, 21, 21, 21, 21, P, P	10	10	50
9	100,000	None	7, 8, 9, 9, 9, 10, 11, 12	1	8	11
10	2,000	"	5, 6, 6, 7, 7, 8, 9, 10, 12	1	9	10
10	400	"	8, 8, 11, 12, 13, P	4	6	40
10	80	"		10	0	100

* Paralyzed on 21st day.

SUMMARY

1. In vitro, chloramphenicol inhibits the growth of Phase I *Hemophilus pertussis* in concentrations of 0.2 to 0.3 μg per ml of medium as determined by the blood agar dilution plate method.

2. Chloramphenicol affords protection following intranasal infection of mice when administered twice daily for one or more days. However, in intracerebral infection, chloramphenicol was much less effective.

REFERENCES

Brownlee, G. and Bushby, S. R. M. 1948, Chemotherapy and pharmacology of aerosporin. Lancet, **254:** 127–132.

Glazko, A. J., Wolf, L. M., Dill, W. A. and Bratton, A. C. Jr. 1949, Biochemical studies on chloramphenicol (chloromycetin) II Tissue distribution and excretion studies. J. Pharmacol. & Exper. Therap. **96:** No. 4, 445–459.

Hegarty, C. P., Thiele, E. and Verwey, W. F. 1945, The in vivo and in vitro activity of streptomycin against *Hemophilus pertussis*. J. Bact. **50:** 651–654.

Kendrick, P. L., Eldering, G., Dixon, M. K. and Misner, J. 1947, Mouse protection tests in the study of pertussis vaccine: A comparative series using the intracerebral route for challenge. Am. J. Pub. Health, **37:** 803–810.

McLean, I. W. Jr., Schwab, J. L., Hillegas, A. B. and Schlingman, A. S. 1949, Susceptibility of micro-organisms to chloramphenicol (chloromycetin). J. Clin. Investigation, **28:** No. 5, 953–963.

EFFECT OF PITUITARY ADRENOCORTICOTROPHIC HORMONE (ACTH) ON EXPERIMENTAL POLIOMYELITIS AND EQUINE ENCEPHALOMYELITIS

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Hormones of the adrenal cortex have recently been shown to exert a protective effect in adrenalectomized rats given a lethal dose of typhoid vaccine.[1] Clinical[2] and experimental evidence[3] indicate that fatigue and chilling during the incubation period lower resistance to poliomyelitis. Also the incidence of paralysis has been shown to be significantly greater during pregnancy.[4,5] These facts suggest that the "alarm reaction" might be involved in the mechanism of nonspecific host resistance to poliomyelitis. Experiments were carried out to study the effect of ACTH on mice inoculated with the Lansing strain of poliomyelitis virus. Similar experiments were also done with mice inoculated with Western equine encephalomyelitis virus.

METHODS

The Lansing strain of poliomyelitis virus and the B.A.I. strain of Western equine encephalomyelitis virus were used. The technique of preparation and storage of mouse brain suspensions of these viruses for intracerebral inoculation is described elsewhere.[6,7] Their identity was verified by neutralization tests with specific hyperimmune antiserums. Two strains of Swiss mice (Webster and Beyer) weighing 13 to 15 g were employed. Groups of mice were inoculated with 10 or 100 LD_{50} units of virus intracerebrally in chemotherapy experiments.

Two types of experiments were done. In the first mice were inoculated twice daily with ACTH beginning 24 hours before virus inoculation, and therapy was continued until onset of signs of infection. In the second mice with paralysis or encephalitis were treated with ACTH, and the survival time was compared with that of control mice. The ACTH was stored at −10 C or in a dry ice cabinet.

RESULTS

Mice were inoculated subcutaneously with 0.75 mg to 1 mg of ACTH in equally divided doses twice daily in all therapy experiments. This amount of drug was apparently not toxic to the mice.

Combined results obtained in three experiments with groups of treated and control mice challenged with approximately 10 and 100 LD_{50} units of the Lansing strain of poliomyelitis virus intracerebrally are shown in table 1.

TABLE 1.—*Effect of ACTH on mice infected with Lansing virus.*

LD_{50} dose	No. of mice	ACTH	Av. incubation period, days	% Mortality
10	24	Treated	12.2±3.6	85
10	26	Control	12.5±3.3	80
100	28	Treated	10.2±2.6	100
100	30	Control	10.0±2.1	100

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1. Lewis, L. A. and Page, I. H. 1949, Ann. New York Acad. Sc. **50**: 547–551.
2. Horstmann, D. M. 1950, J.A.M.A. **142**: 236–241.
3. Levinson, S. O., Milzer, A. and Lewin, P. 1945, Am. J. Hyg. **42**: 204–213.
4. Aycock, W. L. 1941, New England J. Med. **225**: 405–408.
5. Fox, M. J. and Sennett, L. 1945, Am. J. Med. Sc. **209**: 382–387.
6. Milzer, A., Oppenheimer, F. and Levinson, S. O. 1945, J. Immunol. **50**: 331–340.
7. Milzer, A. 1943, Proc. Soc. Exper. Biol. & Med. **54**: 279–282.

ACTH was started 24 hours before virus inoculation and continued daily until onset of paralysis. About half of the treated mice received the drug daily for at least 10 days prior to onset of paralysis. The results shown in table 1 indicate that there was no significant difference in average incubation period or mortality rate in the treated and control mice challenged with 10 and 100 LD_{50} units of virus respectively. In other experiments with comparable numbers of animals in which ACTH therapy was started after onset of paralysis, there was no significant difference in average survival time between the treated and control mice.

Negative results were also obtained in three experiments with groups of mice inoculated intracerebrally with 10 and 100 LD_{50} units of the Western equine encephalomyelitis virus. ACTH therapy was started 24 hours before virus inoculation and given in the same dosage. Results obtained are summarized in table 2. ACTH therapy was also found to have no therapeutic effect when given to mice after onset of signs of encephalitis.

TABLE 2.—*Effect of ACTH on mice infected with Western equine virus.*

LD_{50} dose	No. of mice	ACTH	Av. incubation period, days	% Mortality
10	32	Treated	6.0	92
10	30	Control	6.0	85
100	28	Treated	5.5	100
100	27	Control	5.5	100

DISCUSSION

Under the conditions of the experiments reported here ACTH had no protective or therapeutic effect in mice inoculated with the Lansing strain of poliomyelitis virus or the Western equine encephalomyelitis virus. The amount of ACTH administered to the mice should be adequate, because it was in excess of the usual human therapeutic dosage on a weight basis. Furthermore, ACTH therapy was started 24 hours before virus inoculation and continued daily until onset of paralysis or signs of encephalitis. The dose of ACTH given had no apparent deleterious effect on the mice.

While our experiments were in progress, the publication by Coriell et al[8] appeared in which ACTH was shown to have no beneficial or obvious deleterious effect on the course of poliomyelitis in patients. Treatment was instituted after onset of symptoms, and the daily dosage ranged from 20 to 120 mg.

SUMMARY

Pituitary adrenocorticotrophic hormone (ACTH) was found to have no beneficial effect in either the protection or therapy of mice inoculated with the Lansing strain of poliomyelitis or the B.A.I. strain of Western equine encephalomyelitis.

8. Coriell, L. L., Siegel, A. C., Cook, C. D., Murphy, L. and Stokes, J. Jr. 1950, J.A.M.A. **142**: 1279–1281.

PURIFICATION AND PROPERTIES OF THE AGENT OF FELINE PNEUMONITIS

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Several animal viruses have now been obtained in a highly purified form and their chemical, physical, and morphological properties have been studied intensively (reviewed by Beard, 1948a and b). A number of purification procedures have been devised for the psittacosis-lymphogranuloma group of viruses to produce either satisfactory antigens (Lazarus and Meyer, 1939; McKee et al. 1940; Smadel et al, 1943) or material suitable for electron microscopy (Rake et al, 1946; Hamre et al, 1947; Heinmets and Golub, 1948), but the chemical and physical properties of these agents have not been investigated in detail.

In the present study, the elementary bodies of feline pneumonitis were separated from yolk sac in a considerably purified state as a part of a broad investigation of the properties and host relationships of the psittacosis-lymphogranuloma group of viruses (Weiss, 1950a, b; Gogolak and Weiss, 1950; Moulder and Weiss, 1951a, b; Weiss et al, 1951).

The agent of feline pneumonitis appeared especially well suited for this research. Shortly after its initial isolation from the lungs of cats suffering from pneumonia (Baker, 1942), it was recognized as a typical member of the psittacosis-lymphogranuloma group (Baker, 1944; Hamre and Rake, 1944). Hilleman (1945) and St. John and Gordon (1947) showed by serum neutralization tests, and Hamre and Rake (1944) and Manire and Meyer (1950b) by toxin neutralization tests, that the agent of feline pneumonitis is a distinct agent different from other members of the group. However, it shares with the other psittacosis-lymphogranuloma viruses the ability to elicit a complement fixing antibody (Thomas and Kolb, 1943) and the ability to develop in the yolk sacs of chick embryos and in the lungs of mice (Baker, 1942). Some of its properties place it in an intermediate position among the various agents of the psittacosis-lymphogranuloma group. For example, it resembles murine pneumonitis in its morphology (Weiss, 1949) and in its comparative lack of neuro- and viscerotropism (Baker, 1944). It shares with meningopneumonitis and some of the psittacosis strains a toxin lethal for mice (Hamre and Rake, 1944; Manire and Meyer, 1950a) and an insusceptibility to sulfonamides (Hamre and Rake, 1947).

The ease with which man becomes infected with psittacosis virus has been one of the main handicaps in work with this agent. However, no laboratory infections of feline pneumonitis have been reported, and the agent is generally regarded as nonpathogenic for humans. This is a great advantage in procedures which require repeated handling of concentrated virus suspensions.

The yolk sacs of chick embryos were used as the starting material because the agent of feline pneumonitis develops better in this tissue than in the chorio-

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allantoic cavity (Francis and Gordon, 1945; Hamre et al, 1947). Furthermore, although many agents of the psittacosis-lymphogranuloma group develop well in the chorioallantoic cavity, the yolk sac is probably the best single tissue for cultivation of these agents. Thus, a method for purification of the elementary bodies of feline pneumonitis from yolk sacs might be of more general application to the purification of other members of the group.

MATERIALS AND METHODS

Strain of virus.—The strain of feline pneumonitis virus was obtained in 1943 from Dr. Baker's laboratory and used in many previous investigations (Hilleman, 1945; St. John and Gordon, 1947; Weiss, 1949, 1950a, b). It was preserved in the dry ice box and passed in the yolk sac of chick embryos at various intervals. The record of the exact number of egg passages was no longer available. The strain was highly virulent for chick embryos when inoculated into the yolk sac. A 10% emulsion in 0.25 ml amounts killed embryos in 3 days, a 10^{-4} dilution, in 4 to 5 days, and a 10^{-6} dilution, in 6 days. With lower concentrations some embryos died after 7 days and most of the remaining survived. Probably because of numerous passages in chick embryos, the strain had lost most of its virulence for mice. The undiluted emulsion, when inoculated intranasally into mice, caused only partial consolidation of the lungs. However, the original virulence for mice could be restored by repeated passages. For example, a pool was prepared from the 8th mouse passage which killed mice in about three days. From time to time the identity of the strain was reestablished by a serum neutralization test in which a serum prepared by St. John in 1946 was used.

Titration of virus.—The various steps in the purification procedures were followed by determinations of infectivity. For each titration 40 White Leghorn 6-day-old chick embryos were used. The material to be titrated was diluted in veal infusion broth from 10^{-5} to 10^{-8} and for each dilution 0.25 ml was inoculated into the yolk sac of each of 10 embryos. The eggs were candled once a day and those found dead before the 4th day were discarded. At the end of 10 days the number of LD_{50} per ml fluid tested was calculated according to the method of Reed and Muench (1938). For virus stability experiments only 3 dilutions of virus were inoculated each into 5 eggs. No difficulty was encountered with bacterial contamination. Tubes of thioglycollate broth were inoculated with 0.1 ml aliquots of each specimen. Except for rare instances, no bacterial growth was detected after 5 days of incubation at 37 C.

Staining procedures.—As a check on the purification procedures, the various specimens were stained and examined for the presence of elementary bodies and relative freedom from yolk sac material. The Gram stain was the method of choice. Crystal violet aniline oil was used and the routine procedure was followed except that, as the specimens became more purified, the time of decolorization with 95% ethanol was progressively shortened to two or three seconds. By this procedure, the elementary bodies retained the crystal violet.

Total nitrogen.—The total nitrogen content of the various fractions obtained in purification of the virus was measured by the micro-Kjeldahl technique described by Groman (1951).

Diluents and buffers.—Veal infusion broth, used as a diluent in preparation of yolk sac pools, was prepared from fresh veal and contained in addition 1% peptone and 1% sodium chloride. Phosphate-saline used in the succeeding stages of purification was made up in glass-distilled water and contained 0.128 *M* sodium chloride, 0.005 *M* potassium chloride, 0.001 *M* magnesium sulphate, and 0.01 *M* sodium phosphate buffer, pH 7.4. The phosphate buffer was prepared by addition of hydrochloric acid to sodium hydrogen phosphate. Histidine buffers of pH 5.5 to 9.0 were made from histidine dihydrochloride and sodium hydroxide on the basis of the titration constants given by Greenstein (1931).

Antiserum against normal yolk sac.—Antiserum against normal yolk sac was prepared in rabbits by the intraperitoneal injection of yolk sac pools from uninoculated chick embryos made in the same manner as described for preparation of virus pools. Each rabbit received three 0.5 ml injections of normal yolk sac pool each week for three weeks and was bled by heart puncture one week after the final injection. Serums from 2 to 4 rabbits were pooled and stored at −20 C. On the day of use, the pooled antiserum was inactivated for 30 minutes at 55 C and clarified by centrifugation at 5000 r.p.m. for 60 minutes.

EXPERIMENTAL RESULTS

1. Preparation of purified virus

The agent of feline pneumonitis has been separated from yolk sac and considerably purified by a procedure involving three cycles of high and low speed centrifugation. Normal yolk sac

components are adsorbed on celite during the first cycle of centrifugation and are precipitated with antiserum against normal yolk sac between the second and third cycles. The initial steps in the separation of the agent are similar to those employed by Shepard and Topping (1947) in the preparation of rickettsial antigens. Antiserum against normal host cell antigens has been used in the purification of the virus of silk worm jaundice (Glaser and Stanley, 1943) and the T2, T4 and T6 bacterial viruses (Cohen and Arbogast, 1950).

Purification procedure

All operations were carried out with aseptic precautions. The virus material was always kept in an ice bath except when it was centrifuged at 3 to 5 C.

1. Starting pool.—Seven to 8-day embryos were inoculated in the yolk sac with 0.25 ml of virus emulsion containing about 10^8 LD_{50} doses. The embryos died in about 5 days. The dead eggs were left at 37 C for 6 to 12 hours and then placed at 5 C for another 6 to 12 hours before the yolk sacs were harvested. Yolk sacs harvested immediately after death of the embryo had the highest virus titers (Moulder and Weiss, 1951a), but this procedure of aging the dead eggs before removal of the yolk sacs eliminated much yolk material without greatly reducing the virus titer. The yolk sacs were harvested in pools of 10 into 250 ml centrifuge bottles containing glass beads; twice their weight of veal infusion broth was added; and the bottles were shaken on a mechanical shaker for 30 minutes at room temperature. The yolk sac emulsion was then pipetted into a 50 ml lusteroid tube, capped with a rubber stopper, rapidly frozen and stored in the dry ice box at −70 C for not more than 30 days before use. Each pool of 10 yolk sacs was tested for bacterial contamination and only sterile pools were used. Gram stained smears of this material showed only a few elementary bodies. Many more were probably masked by the great mass of yolk, cell nuclei, and debris present.

2. Waring homogenization.—Four to 5 pools of 10 yolk sacs each were thawed and combined to give a starting pool of about 150 ml. This starting pool was transferred to a Waring blendor, an equal volume of 0.1 *M* sodium phosphate (pH 7.4) was added and the mixture blended for 30 seconds. This step gave little decrease in infectivity.

3. First high speed centrifugation.—Fraction 2 was then centrifuged for 60 minutes at 5000 r.p.m. (2800 *g* at the periphery) in a Servall Type SS-1 angle centrifuge. The supernatant was poured off, and the walls of the centrifuge tubes were wiped with cotton swabs to remove solidified fat. The voluminous sediment was thoroughly suspended in 150 ml phosphate-saline, pH 7.4. The resulting suspension contained about 10% of the original total nitrogen and about 50% of the original infectivity. The lost infectivity could not be found in the discarded supernatant. The number of cell nuclei present in smears of fraction 3 was greatly reduced. Much cell debris was still present, but numerous elementary bodies were clearly seen.

4, 5. Celite adsorption and first low speed centrifugation.—Celite (Johns-Manville) was next added to fraction 3 in the ratio of 0.1 g celite per g yolk sac in the starting pool. The mixture was well-mixed, and visible flocculation occurred within a few minutes. The mixture of celite and virus material was then centrifuged for 15 minutes at 1200 r.p.m. (240 *g* at the periphery) in a horizontal centrifuge (International Clinical Centrifuge, head No. 215). Higher gravitational fields threw down appreciable amounts of virus. The supernatant was removed from the celite sediment and centrifuged again to remove additional celite. Table 1 shows the effect of different amounts of celite on the recovery of infectivity and nitrogen. In a concentration of 0.1 g per g of yolk sac, celite gave excellent purification, but in higher concentrations such as those used by Shepard and Topping (1947), celite removed most of the feline pneumonitis virus.

TABLE 1.—*Effect of celite concentration on yield of infectivity and total nitrogen.*

Celite added in g per g of yolk sac	% of starting pool recovered after adsorption (fraction 5)		Infectivity Index*
	Infectivity	Total nitrogen	
	40	4.4	9
0.05	60	3.2	19
0.10	50	2.2	23
0.20	4	1.4	3

* Infectivity index: % recovery infectivity divided by % recovery total nitrogen.

6, 7. Second high and low speed centrifugation.—Fraction 5 was centrifuged for 60 minutes at 5000 r.p.m. in the angle centrifuge, and the cloudy supernatant was discarded. This supernatant contained about half of the total nitrogen remaining after celite adsorption and none of the infectivity. The sedimented material was gently resuspended in 35 ml phosphate-saline and centrifuged for 15 minutes at 1200 r.p.m. in the

horizontal centrifuge, and a sizeable pellet was discarded. The clarified suspension contained 1% of the initial total nitrogen and 25% of the initial infectivity. In smears of fraction 7, the elementary bodies appeared very clearly in a background of amorphous material. All particulate debris had disappeared.

8. Precipitation of normal components with antiserum against normal yolk sac.—Fraction 7 was next treated with antiserum against normal yolk sac to remove normal egg components. A preliminary titration was performed on each preparation to determine the proper amount of antiserum to be added. Too little antiserum left excess normal antigen in the supernatant; too much antiserum still combined with all the normal antigens but gave a precipitate which could not be packed down at the low speeds at which it was necessary to centrifuge off the immune precipitate. The best ratio of serum to fraction 7 was just in excess of the optimal proportions ratio for fastest flocculation (Boyd, 1947). Table 2 is a sample protocol for determination of the optimal ratio of antiserum to fraction 7 for fastest flocculation and for most satisfactory precipitation of normal antigens.

TABLE 2.—*Selection of optimum amount of antiserum for precipitation of normal egg antigens.*

Ratio of antinormal yolk sac serum to fraction 7	2.00	1.67	1.50*	1.22	1.00	0.80	0.60
Time of flocculation at 37 C in minutes	5	5	3	3	3	4	4
Test for excess antigen in supernatant	0	0	0	±	+	+	+

* Ratio used for removal of normal antigens.

Since antiserum preserved in the frozen state at −20 C had a pH of 7.8 to 8.0 when thawed, the optimal amount of antiserum as determined by preliminary titration was mixed with one half its volume of 0.1 *M* sodium phosphate, pH 7.4, to bring the pH to about 7.5 before it was added to fraction 7. The mixture was allowed to flocculate for 12 to 16 hours at 0 C. The immune precipitate was then removed by centrifuging for 15 minutes at 1200 r.p.m. in the horizontal centrifuge, and the supernatant was set aside. The precipitate was washed twice by suspending in 20 ml phosphate-saline and recentrifuging as above. All supernatants were combined and centrifuged again for 15 minutes at 1200 r.p.m. The washed immune precipitate possessed less than 5% of the infectivity in the combined supernatant and was discarded.

9, 10. Third high and low speed centrifugation.—Fraction 8 was centrifuged in the angle centrifuge for 45 minutes at 5000 r.p.m. The supernatant was thoroughly drained off and discarded. The small, faintly yellow pellet was gently suspended in 2 to 10 ml phosphate-saline and centrifuged 15 to 30 minutes at 1200 r.p.m. in the horizontal centrifuge. The supernatant was removed, and a small pellet was discarded. The final virus suspension contained about 0.15% of the original total nitrogen and about 5% of the original infectivity. Gram stained smears showed that almost all of the amorphous background material present in fraction 7 had been eliminated (fig. 1). Final preparations contained less than 10 bacteria per ml. The virus could be taken through an additional cycle of high and low speed sedimentation without loss of activity but without further purification.

The yield of infectivity and total nitrogen at various stages in purification are summarized in table 3, which includes the results with all preparations made under the conditions just described. The extent of purification has been estimated by the infectivity index which is obtained by dividing the percent recovery of infectivity by the percent recovery of total nitrogen.

It is believed that much of the loss in infectivity during the purification of feline pneumonitis virus is due to inactivation of the elementary bodies, and that the final preparations contained a considerable quantity of inactivated virus. All of the discarded fractions were titrated for infectivity, but none contained more than 5% of the infectivity present in the main virus fraction at that stage of purification. It could not be determined whether the inactive virus was carried proportionally along with the active virus or whether it was mainly eliminated in the discards. It should be remembered that if much inactive virus has been present in a discarded fraction, measurement of infectivity alone would have underestimated the total amount of active and inactive virus discarded.

The chief loss of infectivity occurred

at two different steps in purification. First, half of the infectivity was lost in the first sedimentation of the virus (step 3). This loss cannot now be explained. The supernatant from this sedimentation was virtually inactive, and the purified virus can be centrifuged down and resuspended at least twice under the conditions employed in step 3 without decrease in infectivity. Second, only about 20% of the infectivity was recovered after immune precipitation of normal egg antigens. Again, the discarded fractions were without significant infectivity. The purified virus is stable under the conditions of temperature and pH used in this step. However, it has been found that both normal and immune rabbit serums contain a factor which slowly inactivates purified feline pneumonitis virus at 0 C. The factor is stable for 30 minutes at 55 C and is not associated with the antibody against normal yolk sac. It is probable that this normal serum factor is responsible for at least part of the loss in infectivity during immune precipitation of normal antigens. As discussed later, there is much evidence to show that the immune precipitation brings about great purification of the virus, yet the infectivity index shows a surprisingly small rise in this step. This is further evidence for the belief that the chief cause for loss of infectivity in the immune precipitation of normal antigens is the inactivation of elementary bodies present in the final purified preparation.

TABLE 3.—*Yield of infectivity and total nitrogen at various stages in purification of feline pneumonitis virus.*

Fraction	Description	No. of samples	Infestivity in fraction as % of starting pool*			Total nitrogen in fraction as % of starting pool*			Infectivity index		
			High	Low	Mean	High	Low	Mean	High	Low	Mean
3	1st. high speed sediment	5	160	25	59	11.0	3.7	7.7	26	5	8
7	2nd. low speed supernatant	8	52	14	25	1.35	0.22	0.79	73	14	32
10	3rd. low speed supernatant	7	8	1	5	0.37	0.05	0.13	73	16	38

* The starting pools contained an average of 6.0 mg N per ml. of which about 20% came from the veal infusion broth, and an average of $10^{7.3}$ LD_{50} per ml.

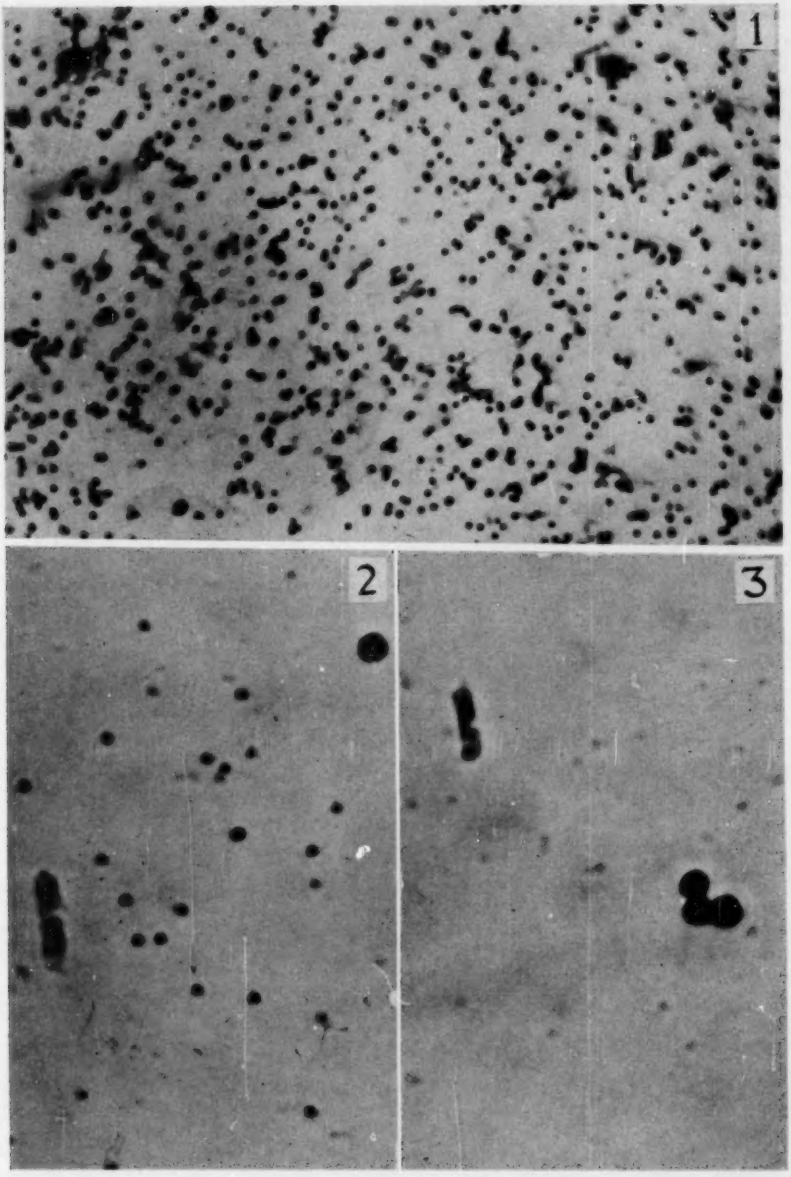
2. *Properties of the purified virus*

Morphological properties.—As previously noted (Weiss, 1950a), the agent of feline pneumonitis retains the crystal violet in the routine Gram stain if the time of ethanol decolorization is limited to only a few seconds. Figure 1 shows a Gram stained smear of purified feline pneumonitis virus which was decolorized with 95% ethanol for 2 seconds. The deeply stained material appears to consist entirely of elementary bodies of the agent. A small amount of amorphous, lightly stained material is also visible and may represent normal yolk sac material present in the final preparation. In order to estimate the relative degree of Gram positiveness of the elementary bodies, the purified virus was mixed with young cultures of *Staphylococcus albus* and *Escherichia*

FIGURES 1–3 are light microscope photographs of purified elementary bodies of feline pneumonitis (fraction 10). The photographs were taken by Mr. J. W. Crunelle of the Photographic Department of the University of Chicago Clinics.

FIG. 1.—Gram stain, decolorization 2 seconds. ×2000. The virus suspension from which this smear was made contained 0.028 mg nitrogen per ml.

FIGURES 2, 3.—Gram stain, decolorization 5 and 20 seconds, respectively. The elementary bodies still appear intensely stained after 5 seconds and are decolorized after 20 seconds. Note the intensely stained *Staph. albus* and decolorized *E. coli*. ×4000.



FIGS. 1–3

coli, and smears of the mixed suspension were stained with Gram stain and decolorized with 95% ethanol for different times. After a 5 second decolorization, *E. coli* was Gram negative while the elementary bodies and *Staph. albus* were Gram positive (fig. 2). However, after a 20 second decolorization, the elementary bodies were also Gram negative, while *Staph. albus* was still positive (fig. 3). It thus appears that the elementary bodies of feline pneumonitis are weakly Gram positive.

Concentrated suspensions of purified virus (0.3 to 0.4 mg *N* per ml) are weakly stained in the Feulgen reaction and are somewhat more strongly stained by the pyronine portion of the methyl green pyronine reagent, suggesting the presence of both desoxyribonucleic and ribonucleic acids in the elementary bodies.*

Electron micrographs of virus preparations before immune precipitation of normal components show the elementary bodies enmeshed in a gelatinous appearing matrix of high density, and individual elementary bodies are seen only with difficulty (fig. 4). Such a matrix has also been described by Rake et al (1946) and Hamre et al (1947). After purification by immune precipitation, the elementary bodies stand out clearly and only traces of the matrix substance remain (fig. 5). Figure 6 shows the elementary bodies in higher magnification. At this magnification, a small amount of nonvirus material of a much smaller size than the elementary bodies themselves is seen in all preparations. As described by Hamre et al (1947), the elementary bodies of feline pneumonitis appear to be composed of at least two components. One is a central mass of high density often having a complexly folded and convoluted surface. The other component is located on the periphery of the elementary bodies, is of lesser density, and has the appearance of a containing membrane. A size distribution was obtained from measurement of the diameter and height of 50 elementary bodies selected at random from electron micrographs of 3 different preparations. The mean diameter of the platinum-palladium shadowed bodies was 423 mμ with a standard error of 6.1%. The height of the elementary bodies as estimated from the length of the shadows and the angle at which the shadowing metal was cast was 161 mμ with a standard error of 9.3%. These dimensions are somewhat smaller than those reported by Hamre et al (1947) for the size of gold shadowed elementary bodies of feline pneumonitis obtained from allantoic fluid.†

* The results of these tests were kindly confirmed by Drs. P. P. H. DeBruyn and Richard Farr of the Department of Anatomy, The University of Chicago.

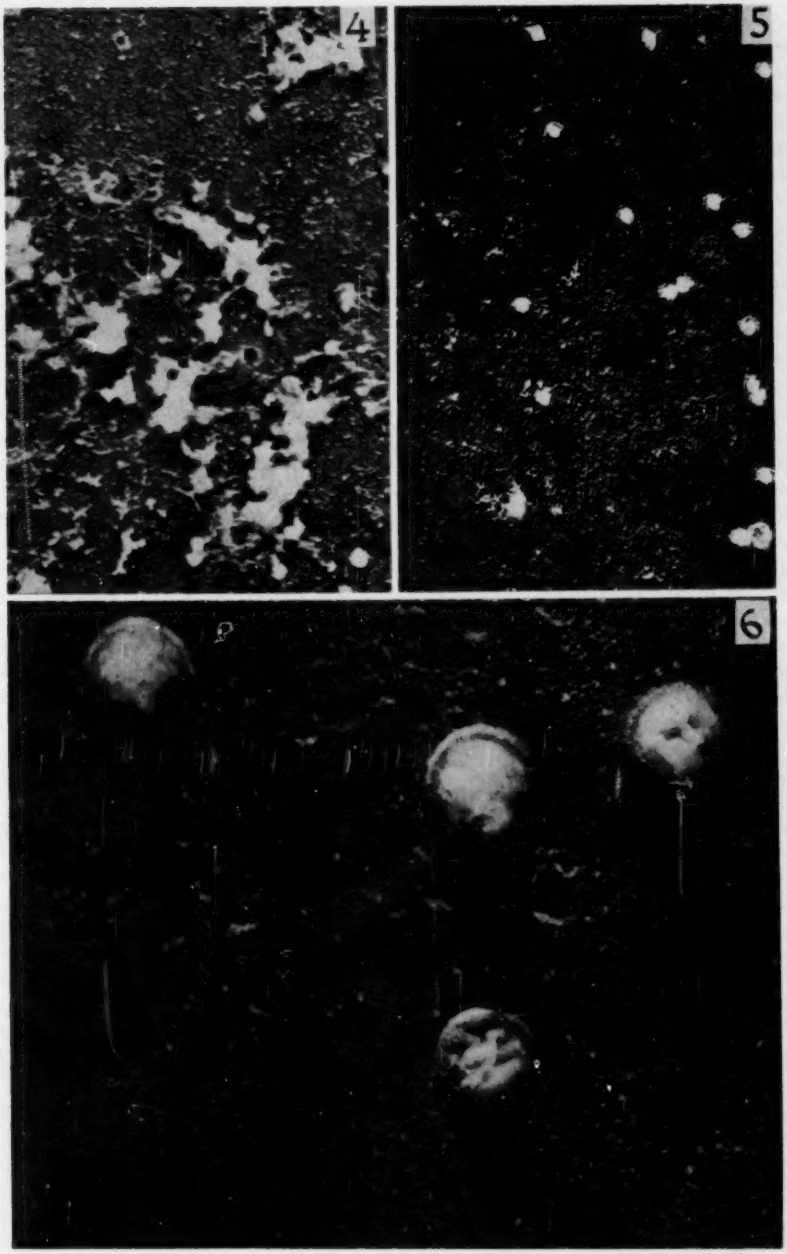
† The electron micrographs were taken at the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind., through the kind cooperation of Mr. R. A. Kern of the Department of Physical Chemistry.

FIGURES 4–6 are electron micrographs of the agent of feline pneumonitis. Virus suspensions in phosphate-saline were dried on formvar screens, washed twice with sterile distilled water to remove salts, and shadow-cast in a high vacuum at an angle of 11° with platinum-palladium from a tungsten filament. Electron micrographs were taken with an RCA Model B electron microscope at a magnification one-third that of the final magnification shown here.

FIG. 4.—Partially purified preparation before immune precipitation of normal components (fraction 7). ×7500. The suspension contained 0.180 mg nitrogen per ml.

FIG. 5.—Final preparation after immune precipitation of normal components (fraction 10). ×7500. The suspension contained 0.022 mg nitrogen per ml.

FIG. 6.—Final preparation after immune precipitation of normal components (fraction 10). ×39,000. This preparation was identical with that shown in fig. 1.



FIGS. 4–6

Behavior in the ultracentrifuge.—Three final virus preparations containing 0.15 to 0.30 mg nitrogen per ml and suspended in phosphate-saline, pH 7.4 and 0.15 ionic strength, were examined in the Spinco Model E analytical ultracentrifuge. In a centrifugal field of 715 *g* at the center of the cell, the preparations showed a diffuse sedimentation boundary with an s_{20} of about 10,000 Svedberg units, about that expected from the size of the elementary bodies in electron micrographs. The heterogeneity of the purified virus as revealed by sedimentation analysis was considerably greater than would have been predicted from the size distribution of the elementary bodies obtained from electron micrographs. However, since the preparations showed remarkably low refractive index gradients for their nitrogen content, and since the rotor showed some vibration at the extreme low speed at which the analyses were made, at least a portion of the observed heterogeneity was probably due to these unfavorable conditions of study.*

Immunological properties.—Since the agent of feline pneumonitis was purified by immune precipitation of normal egg components, the final preparation should be expected to contain only minimal quantities of normal antigens. However, all final preparations of virus were tested directly for the presence of normal antigens. When a normal antigen preparation, corresponding to virus fraction 7, was reacted in increasing dilutions with antiserum against normal yolk sac, as little as 0.003 mg nitrogen per ml of normal antigen gave a definite precipitate both in the interfacial ring test and in the single phase flocculation test. When final virus suspensions containing 0.05 to 0.10 mg nitrogen per ml were tested with normal antiserum under the same conditions, no precipitate was formed. Therefore, it can be estimated that the virus suspensions contained less than 3 to 6% normal, precipitable egg antigens ($0.003/0.05-0.10\times100=3-6\%$). However, this does not indicate the absence of nonprecipitable impurities.

The identity of the infectivity in the purified material with the infectivity present in the starting emulsion was established by a serum neutralization test performed in chick embryos as described by Weiss (1950a). The immune serum was obtained from a rooster which had been injected intraperitoneally with 0.5 ml starting pool (fraction 1) thrice weekly for a period of 14 weeks.

Stability of the virus.—In the usual phosphate-saline medium, the purified virus can be frozen at −70 C and thawed immediately without detectable loss of infectivity. The effect of storage of the virus at −70 C upon its infectivity is shown in figure 7. When the logarithm of the infectivity, expressed as the percent of the original titer, is plotted against the time of storage at −70 C, the experimental points fall on a straight line, indicating that the infectivity declines according to first order reaction kinetics. This straight line gives an apparent half-life of 30 days for the virus at −70 C. The infectivity of purified feline pneumonitis virus remains unchanged for at least 24 hours at 0 C (fig. 8). At 37 C, the infectivity rapidly decreases according to first order reaction kinetics, and the best straight line drawn through the experimental points indicates an apparent half-life of 90 minutes for virus infectivity at 37 C. It is of interest to compare the half-life of the purified virus at 37 C with the half-life of 12 hours obtained for the virus in intact chick embryos in which it is believed virus reproduction has ceased (Gogolak

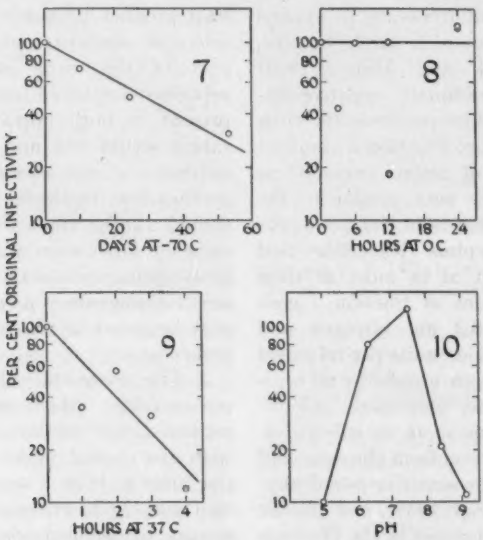
* We wish to thank Dr. Frank W. Putnam of the Department of Biochemistry, The University of Chicago, for carrying out the ultracentrifuge analysis of the virus preparations.

and Weiss, 1950; Moulder and Weiss, 1951a).

To measure the effect of the inorganic ions of the phosphate-saline medium, sodium chloride, potassium chloride, magnesium sulphate, and sodium phosphate, upon the stability of the purified virus, a final virus preparation was twice centrifuged down and resuspended in distilled water at 0 C. The infectivity of the virus suspended in distilled water was only a little less than that of a control virus sample twice sedimented and resuspended in phosphate-saline. Therefore, it was concluded that the inorganic ions of the medium do not materially increase the stability of the purified virus at 0 C.

At pH 7.4 and 0 C, the infectivity of purified feline pneumonitis virus is equally stable in saline buffered with phosphate, glycyl-glycine, and histidine. To determine the effect of pH upon the infectivity of the virus, the purified material was suspended in balanced saline buffered with 0.01 *M* histidine from pH 5.5 to 9.0 and kept for 24 hours at 0 C. Figure 10 shows the infectivity remaining at the end of this period. The infectivity of the virus remained unaltered from pH 6.5 to 7.5 and was rapidly destroyed outside this pH range. The elementary bodies did not spontaneously agglutinate at any pH tested.

Metabolic properties.—Although one of the chief objectives in the purification of the agent of feline pneumonitis has



FIGURES 7–10 show the effect of temperature and pH on the infectivity of feline pneumonitis virus. All experiments were carried out with a single lot of purified virus (fraction 10). In each test, the initial infectivity was $10^{6.5}LD_{50}$ per ml and the nitrogen concentration was 0.07 mg per ml.

FIG. 7.—Effect of storage at −70 C upon virus infectivity.

FIG. 8.—The effect of maintenance of 0 C upon virus infectivity.

FIG. 9.—The effect of maintenance at 37 C upon virus infectivity.

FIG. 10.—The effect of pH upon virus infectivity. The virus was kept at 0 C for 24 hours in saline buffered with 0.01 *M* histidine at the designated pH.

been the study of the possible independent metabolism of this agent, systematic metabolic investigations have been postponed until adequate characterization of the purified virus has been achieved. However, the recent discovery of an independent metabolism of glutamate and pyruvate in typhus rickettsiae (Bovarnick and Snyder, 1949; Bovarnick and Miller, 1950) makes some preliminary negative observations on feline pneumonitis virus worthy of mention. Fraction 7 obtained in purification of feline pneumonitis virus is probably very similar to the type of preparation from chick embryos infected with typhus rickettsiae used by Bovarnick et al in most of their work. Preparations of fraction 7 containing up to 0.3 mg nitrogen and $10^{7.8}$ LD_{50} infectious units per ml failed to show any oxygen uptake at all with glucose, pyruvate, glutamate, α-ketoglutarate, and succinate as substrates. No lactate formation from glucose could be detected by the sensitive p-hydroxydiphenyl test (Feigl, 1939), and glucose was not phosphorylated in the presence of adenosine triphosphate. Under similar conditions, Bovarnick et al (1949, 1950) observed a definite oxidation of pyruvate and especially of glutamate by typhus rickettsiae. Assuming that typhus rickettsiae and feline pneumonitis virus multiply to approximately the same extent in the yolk sacs of chick embryos, the agent of feline pneumonitis oxidizes pyruvate and glutamate at a much slower rate, if at all.

SUMMARY AND CONCLUSIONS

1. The agent of feline pneumonitis was separated from the yolk sacs of chick embryos and purified by a method involving three cycles of high and low speed centrifugation. Normal yolk sac components were adsorbed on celite during the first cycle of centrifugation and were precipitated with antiserum against normal yolk sac between the second and third cycles. The final virus suspension contained about 0.15% of the original total nitrogen and about 5% of the original infectivity. Loss of virus was due in part to inactivation, and the final product probably contained considerable noninfectious virus. A large part of the virus inactivation was probably due to an inactivating factor present in both normal and immune rabbit serum and not associated with antibody against normal yolk sac. The purification methods described here should also be applicable to the purification of other members of the psittacosis-lymphogranuloma group and of other infectious agents of a similar size which may be grown in the yolk sacs of chick embryos.

2. The elementary bodies of feline pneumonitis, when stained by the routine Gram method, intensely stain with the crystal violet and retain the dye after at least 5 seconds decolorization with 95% ethanol. Gram stained smears of purified virus preparations show the presence of large numbers of elementary bodies and only a small amount of amorphous nonvirus material.

3. Electron micrographs of the purified virus also show only small quantities of nonvirus material. In platinum-palladium shadowed preparations, the elementary bodies appear to be composed of a dense central material and a less dense peripheral substance. The mean diameter of the shadowed elementary bodies was 423 mμ, and their height was 161 mμ.

4. Analysis of the purified virus in the ultracentrifuge showed the presence of a diffuse sedimenting boundary with an s_{20} of about 10,000 Svedberg units. The heterogeneity of the preparations as indicated by sedimentation analysis

was greater than the heterogeneity of particle size of the elementary bodies in electron micrographs.

5. Final virus suspensions did not give precipitates with rabbit antiserum against normal yolk sac under conditions in which a contamination with as little as 3% normal antigen nitrogen should have been detected.

6. The final virus suspensions lost their infectivity very slowly when stored at −70 C. Their infectivity remained unchanged for at least 24 hours at 0 C between pH 6.5 and 7.5 and was not appreciably reduced by repeated sedimentation at this temperature. The virus was rapidly inactivated at 37 C.

7. Purified feline pneumonitis virus did not consume oxygen in the presence of a number of substrates, did not form lactate from glucose, and did not phosphorylate glucose in the presence of adenosine triphosphate.

In conclusion, the elementary bodies of feline pneumonitis have been separated from the yolk sacs of chick embryos in a state of comparative purity. Precipitable normal tissue antigens are absent, although there is evidence for the presence of some other nonvirus contaminants. The stability of the best preparations so far obtained is sufficient to allow still further purification. The most serious objection to the present procedure is the inactivation of the virus during the immune precipitation of normal egg antigens. However, since this inactivation is due to a factor not associated with the antibody against normal yolk sac, it should be possible to separate the precipitating antibody from the inactivating factor.

REFERENCES

Baker, J. A. 1942, Science, **96**: 475–476.

———. 1944, J. Exper. Med. **79**: 159–172.

Beard, J. W. 1948a, J. Immunol. **58**: 49–108.

———. 1948b, Physiol. Rev. **28**: 349–367.

Bovarnick, M. R. and Snyder, J. C. 1949, J. Exper. Med. **89**: 561–565.

Bovarnick, M. R. and Miller, J. C. 1950, J. Biol. Chem. **184**: 661–676.

Boyd, W. C. 1947, Fundamentals of Immunology, ed. 2, New York, Interscience Press, pp. 246–250.

Cohen, S. S. and Arbogast, R. 1950, J. Exper. Med. **91**: 607–618.

Feigl, F. 1939, Qualitative analysis by spot tests, New York, Nordemann, p. 333.

Francis, R. D. and Gordon. F. B. 1945, Proc. Soc. Exper. Biol. & Med. **59**: 270–272.

Glaser, R. W. and Stanley, W. M. 1943, J. Exper. Med. **77**: 451–466.

Gogolak, F. M. and Weiss, E. 1950, J. Infect. Dis. **87**: 264–274.

Greenstein, J. P. 1931, J. Biol. Chem. **93**: 479–494.

Groman, N. B. 1951, J. Infect. Dis. **88**: In press.

Hamre, D. and Rake, G. 1941, J. Infect. Dis. **74**: 206–211.

———. 1947, J. Infect. Dis. **81**: 175–190.

Hamre, D., Rake, H. and Rake G. 1947, J. Exper. Med. **86**: 1–6.

Heinmets, F. and Golub, O. J. 1948, J. Bact. **56**: 509–525.

Hilleman, M. R. 1945, J. Infect. Dis. **76**: 96–114.

Lazarus, A. S. and Meyer, K. F. 1939, J. Bact. **38**: 121–151.

Manire, G. P. and Meyer, K. F. 1950a, J. Infect. Dis. **86**: 226–232.

———. 1950b, J. Infect. Dis. **86**: 241–250.

McKee, C. M., Rake, G. and Shaffer, M. 1950, Proc. Soc. Exper. Biol. & Med. **44**: 410–413.

Moulder, J. W. and Weiss, E. 1951a, J. Infect. Dis. **88**: 68–76.

———. 1951b, J. Infect. Dis. **88**: 77–80.

Rake, G., Rake, H., Hamre, D. and Groupe, V. 1946, Proc. Soc. Exper. Biol. & Med. **63**: 489–491.

Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27**: 493–497.

Shepard, C. C. and Topping, N. H. 1947, J. Immunol. **55**: 97–102.

Smadel, J. E , Wertman, K. and Reagan, R. L. 1943, Proc. Soc. Exper. Biol. & Med. **65**: 70–74.

St. John, E. and Gordon, F. B. 1947, J. Infect. Dis. **80**: 297–306.

Thomas L. and Kolb, E. M. 1943, Proc. Soc. Exper. Biol. & Med. **54**: 172–174.

Weiss, E. 1949, J. Infect. Dis. **84**: 125–149.

———. 1950a, J. Infect. Dis. **86**: 27–32.

———. 1950b, J. Infect. Dis. **87**: 249–263.

Weiss, E., Moulder, J. W. and Itatani, M. K. 1951, J. Infect. Dis. In press.

RESPIRATORY METABOLISM OF THE EXTRA-EMBRYONIC MEMBRANES OF CHICK EMBRYOS IN RELATION TO MULTIPLICATION OF THE AGENT OF FELINE PNEUMONITIS

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The respiratory metabolism of the extra-embryonic membranes of chick embryos and the effect of infection with the agent of feline pneumonitis upon their metabolism have been studied as a part of a general investigation of the relation between reproduction of members of the psittacosis-lymphogranuloma group of viruses and host cell metabolism (Weiss, 1950a; Moulder and Weiss, 1951b; Weiss et al, 1951). Since it has been demonstrated that feline pneumonitis virus (Weiss, 1950a), as well as typhus rickettsiae (Rabinowitz et al, 1948) and influenza virus (Lahelle and Horsfall, 1949), may be cultivated in the extra-embryonic membranes of chick embryos killed by chilling, the respiratory metabolism of membranes from dead embryos has also been studied.

MATERIALS AND METHODS

Determination of virus reproduction.—The strain of feline pneumonitis virus and most of the chick embryo procedures used here are described in the preceding publication (Moulder and Weiss, 1951a). The only important departure from these procedures consisted in the use of whole eggs for determination of virus growth. In early experiments, it was noticed that most of the yolk was eliminated from the yolk sacs of embryos within a few hours after they died from feline pneumonitis infection. This change in composition and weight of the yolk sac introduces a serious error when the virus titer in yolk sacs of living embryos is compared with the titer in dead ones. In later experiments, this error was eliminated by determining the virus content of whole eggs. For each titration, the entire contents of 4 eggs were placed in a Waring blendor, blended for 45 seconds, and the homogenate thus prepared was tested for virus content by the procedure previously described (Moulder and Weiss, 1951a). The titer of virus was then expressed as the number of LD_{50} per egg. Histological sections of yolk sac were prepared as described by Weiss (1950b).

Measurement of the metabolism of extra-embryonic membranes.—The yolk sac of chick embryos is well suited for direct manometric study without slicing or mincing. The yolk sac is composed of a single layer of entodermal cells which enlarges by formation of folds and villi. It is associated only with blood vessels of various sizes and a few supporting elements. Although some of the entodermal cells of the chorioallantois become infected with feline pneumonitis virus inoculated into the yolk sac, the bulk of the infection occurs in the yolk sac itself. In microscopic sections, the virus is first detected about 36 hours before the death of the embryos, and the number of vesicles present increases very rapidly. At the time of death, each entodermal cell of the yolk sac is usually infected with 3 or 4 vesicles, clusters, or initial bodies.

For respiratory measurements, each egg was opened and its contents poured into a Petri dish. The extra-embryonic membranes were removed as completely as possible, washed 3 to 5 times in cold 0.9% sodium chloride to remove as much yolk as possible, and kept in cold 0.9% sodium chloride until used. Since it was difficult to separate the chorioallantois from the yolk sac in embryos which had been dead for some time, it appeared desirable to include all the extra-embryonic membranes in this study. Up to this point, all operations were carried out under aseptic conditions. Subsequent operations were

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carried out as cleanly as possible but not with strictly aseptic technique. The membranes were blotted as dry as possible on soft filter paper, and 200 to 400 mg portions were transferred to squares of hardened filter paper and weighed to the nearest mg on a torsion balance. The weighed tissues were then placed in appropriate Warburg flasks. The extra-embryonic membranes of chick embryos killed by chilling on the 6th day weighed 200 to 400 mg, and the whole tissue was placed in the Warburg flask. The weight of the membranes from living embryos increased with the age of the embryo and exceeded 1 g by the 12th day. Portions of suitable weight were cut at random from these large membranes.

The respiratory metabolism of the extra-embryonic membranes was measured in terms of oxygen consumption, expressed as Q_{O_2},* and anaerobic glycolysis, expressed as $Q_{CO_2}^{N_2}$.† Oxygen uptake and carbon dioxide production were determined in 9 ml flasks by the direct method of Warburg (Dixon, 1943). No retention factor was used in calculation of carbon dioxide evolution. For oxygen uptake, extra-embryonic membranes were weighed into flasks containing 1.0 ml Krebs phosphate-saline, pH 7.4 (Krebs and Henseleit, 1932), with 0.01 *M* added glucose and 0.1 ml 20% potassium hydroxide absorbed on a filter paper roll in the center cup. The gas phase was 100% oxygen. For carbon dioxide production, the flask was filled with 1.0 ml Krebs calcium-free bicarbonate saline (Krebs and Henseleit, 1932) containing 0.01 *M* glucose and 0.02 *M* bicarbonate to give a pH of 7.4 in 5% carbon dioxide at 37 C. The gas phase was 5% carbon dioxide and 95% nitrogen. All flasks were equilibrated for 15 minutes at 37 C, and gas exchange was measured for one hour. The oxygen uptake and carbon dioxide evolution of the membranes were proportional to the weight of tissue taken and declined only slightly during the period of observation.

At the end of the hour, the membranes were removed from the flasks for determination of dry weight, and the flask contents were streaked on blood agar plates to estimate the degree of bacterial contamination. Most plates showed only scattered colonies of bacteria, and the few heavily contaminated samples were discarded.

The dry weight of the extra-embryonic membranes of chick embryos is about 20% of the weight of the fresh wet tissues from living embryos and about 10% of the wet weight of membranes from embryos killed by chilling. Of this dry weight, 50% to 75% may be extracted with di-ethyl ether. Consistent values for Q_{O_2} and $Q_{CO_2}^{N_2}$ could not be obtained with the total dry weights of the membranes, but good reproduction was achieved when the ether-insoluble dry weights were used in calculation of the Q values. Therefore, the following procedure was routinely used to determine the ether-insoluble dry weights of the extra-embryonic membranes, and all values reported are based on dry weights obtained by this method. The membranes were removed from the Warburg flasks and drained on filter paper. They were then placed in 10 mm microcrucibles and dried one hour at 105 C. This firmly cemented the tissue to the bottom of the crucible. The crucibles were then placed in 1 oz screw cap bottles containing 15 ml ether and allowed to stand overnight at room temperature. The ether was removed by suction and a fresh 15 ml portion of ether was added. After 4 more hours at room temperature, the ether was removed, and the crucibles were dried briefly under reduced pressure to remove all the ether and then at 105 C for one hour. As determined in this manner, the ether-insoluble dry weights of the extra-embryonic membranes of both living and dead embryos were about 5% of the wet weights of the fresh membranes. No change in either the total dry weight or the ether-insoluble dry weight was noted in membranes from chick embryos infected with feline pneumonitis virus.

EXPERIMENTAL RESULTS

Metabolism of the extra-embryonic membranes of living chick embryos.—The oxygen consumption (Q_{O_2}) and the anaerobic glycolysis ($Q_{CO_2}^{N_2}$) of the extraembryonic membranes from 14 chick embryos 5 to 13 days of age were determined. There was no apparent relationship between the metabolic rates of the membranes and the age of the embryos from which they were taken, and the mean values of Q_{O_2} and $Q_{CO_2}^{N_2}$ for the entire 14 pairs of observations are given in table 1. It is believed that these Q values, based on the ether-insoluble

* Q_{O_2} = microliters oxygen consumed in one hour by 1 mg dry tissue.

† $Q_{CO_2}^{N_2}$ = microliters carbon dioxide liberated under anaerobic conditions from bicarbonate buffered medium in one hour by one mg dry tissue. In tissues of higher animals, this value is approximately equal to the amount of lactic acid formed from glucose and is therefore a measure of the extent of anaerobic glycolysis.

TABLE 1.—*Metabolism of extra-embryonic membranes from uninoculated living chick embryos and from living chick embryos inoculated with the agent of feline pneumonitis.*

Treatment of embryo	Q_{O_2}	$Q_{CO_2}^{N_2}$
Uninoculated	5.6±0.4 (14)	5.0±0.4 (14)
Inoculated with feline pneumonitis virus	4.6±0.4 (13)	5.0±0.4 (13)

Each Q value is expressed as the mean ± standard error of the mean (number of observations).

dry weights of the extra-embryonic membranes, give a reasonably valid estimate of the metabolic rates of the active cellular elements in the membranes. The high glycolytic rate is characteristic of embryonic tissues.

To study the effect of infection with feline pneumonitis virus upon the respiratory metabolism of extra-embyronic membranes, 13 chick embryos were inoculated with 10^2 to 10^5 LD_{50} of yolk sac emulsion as determined by the chick embryo titration described previously (Moulder and Weiss, 1951a). The metabolism of their extra-embryonic membranes was measured 1 to 7 days later while the embryos were still alive. Although most of these specimens contained large numbers of elementary bodies as seen in stained smears, the rates of oxygen consumption and anaerobic glycolysis did not differ significantly from those of membranes from uninoculated embryos (table 1).

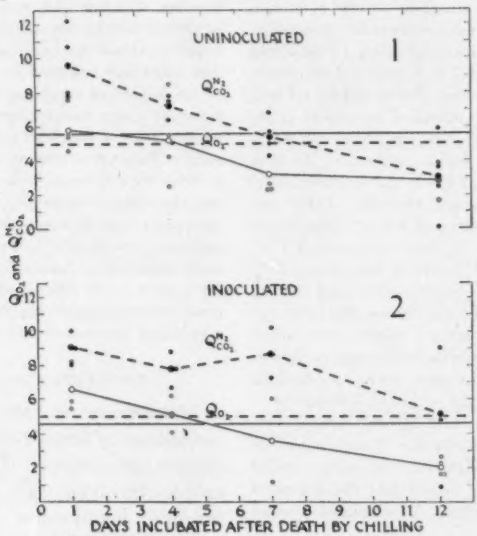

FIG. 1.—Metabolism of the extra-embryonic membranes from uninoculated chick embryos killed by chilling. Individual observations are shown by small circles; the mean value of observations taken on a given day, by a large circle. Q_{O_2} is represented by open circles, and the mean values are connected by a solid line. $Q_{CO_2}^{N_2}$ is represented by solid circles, and the mean values are connected by a broken line. The solid and broken lines parallel to the horizontal axis represent, respectively, the mean Q_{O_2} and $Q_{CO_2}^{N_2}$ of living, uninoculated embryo membranes taken from table 1.

FIG. 2.—Metabolism of the extra-embryonic membranes from chick embryos killed by chilling and inoculated with the agent of feline pneumonitis. The notation is the same as in figure 1. The lines parallel to the horizontal axis represent the mean $Q_{O_2}Q_{CO_2}^{N_2}$ of extra-embryonic membranes from living embryos inoculated with feline pneumonitis virus (table 1).

Metabolism of the extra-embryonic membranes from chick embryos killed by chilling.—When chick embryos were killed by chilling and reincubated at 37 C, the agent of feline pneumonitis multiplied in the yolk sacs of these embryos for at least 7 days after their death (Weiss, 1950a). Investigation of the respiratory metabolism of the extra-embryonic membranes of dead embryos revealed that during this period, oxygen uptake and anaerobic glycolysis proceeded at near normal rates (fig. 1). Since the Q_{O_2} and $Q^{N_2}_{CO_2}$ of the extra-embryonic membranes of living embryos did not vary with age of the embryo, the mean values shown in table 1 have been taken as the best available baseline for comparison of the metabolic rates in dead and living embryos. Consistent Q values were not obtained until 24 hours after incubation of the dead embryos at 37 C. At that time, both the Q_{O_2} and the $Q^{N_2}_{CO_2}$ were above normal, the $Q^{N_2}_{CO_2}$ being greatly elevated. Both rates slowly declined to about the normal rate for living embryos in 4 to 7 days, and even after 12 days both the oxygen uptake and anaerobic glycolysis of the extra-embryonic membranes from dead embryos were still greater than 50% of normal. These findings are in harmony with the observation that viable cells may be demonstrated in such dead embryos up to 16 days after death (Bucciante, 1931; Rabinowitz et al, 1948). The temporary rise in the metabolic rate of the membranes after death of the embryos is not readily explained. It may be another example of the general phenomenon of initial stimulation following cell injury, due perhaps to loss of controlling mechanisms operative in the intact embryos.

The effect of infection with feline pneumonitis virus upon the respiratory metabolism of membranes from dead embryos was studied in another series of dead chick embryos which were inoculated with 10^5 LD_{50} doses of this agent 4 hours after reincubation at 37 C. Examination of stained smears from the membranes of the embryos showed that the virus grew actively. Oxygen uptake and anaerobic glycolysis of the extra-embryonic membranes of the dead

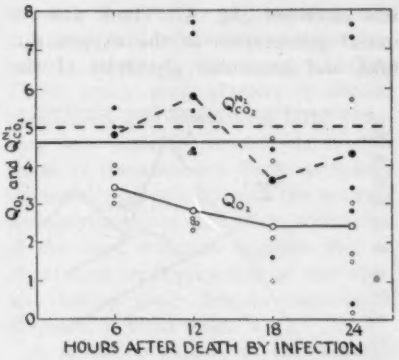

FIG. 3.—Metabolism of the extra-embryonic membranes of chick embryos killed by infection with the agent of feline pneumonitis. The notation is as in figure 1. The lines parallel to the horizontal axis have the same significance as in figure 2.

infected embryos were followed for 12 days (fig. 2). The Q_{O_2} and $Q^{N_2}_{CO_2}$ of the infected dead membranes fell off at about the same rate as in uninoculated dead membranes. The data suggest that the rate of anaerobic glycolysis may drop more slowly in infected membranes, but the difference between such small numbers of observations is not statistically significant.

Metabolism of the extra-embryonic membranes from chick embryos killed by infection with the agent of feline pneumonitis.—As shown in table 1, infection with feline pneumonitis virus did not significantly alter the rate of oxygen uptake and anaerobic glycolysis in the extra-embryonic membranes of living

embryos. Figures 1 and 2 indicate that the respiratory metabolism of membranes from embryos killed by chilling was initially higher than normal and then declined very slowly over a period of many days. However, when embryos died 3 to 4½ days after inoculation with 10^4 to 10^6 LD_{50} doses of feline pneumonitis virus, an entirely different result was obtained (fig. 3). There was no initial acceleration in the oxygen uptake and anaerobic glycolysis of the

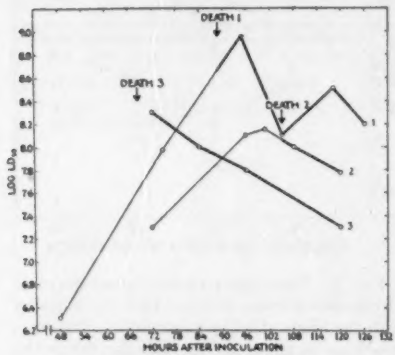

FIG. 4.—Relation of death of the embryo to multiplication of feline pneumonitis virus. Series 1. Eggs were inoculated with $10^{4.8}$ LD_{50} doses, and after 89 hours all living embryos were discarded, and all subsequent observations were made on embryos dead between 83 and 89 hours. The virus titer is expressed as LD_{50} per egg. Series 2. 10^4 LD_{50} inoculum. Dead embryos used in this series died between 98 and 104 hours. The virus titer is expressed as LD_{50} per yolk sac. Series 3. 10^6 LD_{50} inoculum. Dead embryos used in this series died between 62 and 68 hours. The virus titer is expressed as LD_{50} per egg.

extra-embryonic membranes after death of the embryos. Instead, the oxygen uptake fell so rapidly that in one day the Q_{O_2} dropped to a level lower than that obtained in embryos 7 days after death by chilling (compare fig. 2 and 3, with 4). The mean anaerobic glycolysis one day after death by infection was also less than that found 7 days after death by chilling. However, in this series of observations, the mean $Q^{N_2}_{CO_2}$ does not give a complete picture of what occurred. Of 13 observations of anaerobic glycolysis in the 12 to 24 hour period following death of the embryos, only 5 observations were within 20% of the mean value for living embryos, while 4 were elevated at least 50% above the normal mean, and 3 were at least 50% below. This variation, which is much greater than that encountered in measurement of the $Q^{N_2}_{CO_2}$ of membranes from living embryos, suggests that death by infection may, depending upon some unknown factor, cause either an increase or a decrease in the rate of anaerobic glycolysis.

Since it had already been found that the virus of feline pneumonitis multiplies in the yolk sacs of chick embryos killed by chilling (Weiss, 1950a), in which the rate of oxygen uptake is slowly declining, it was of interest to learn whether the virus can also reproduce in the membranes of embryos killed by infection, in which the oxygen uptake is declining much faster. Therefore, at the same time embryos were inoculated for the metabolic experiments described in the preceding paragraph, other embryos were similarly inoculated for determination of the relationship of virus multiplication to death of the embryo. At suitable intervals before and after death of the embryos, groups of 4 eggs were selected at random and their entire contents pooled and titrated for virus content as described earlier. With a single exception,* virus multi-

* In series 1 (fig. 4), after an initial sharp drop after death of the embryo, the virus titer rose between 105 and 120 hours after inoculation. However, the titer again fell between 120 and 126 hours, and at 167 hours (not shown in fig. 4) the titer had fallen to $10^{6.7}$, almost exactly the value predicted if the titer continued to fall at the same terminal rate indicated in figure 4.

plication ceased with the death of the embryos in each of three independent series of experiments (fig. 4). In each series, the highest virus titer was obtained in the pool taken closest to the time of death of the embryos. The concentration of infective virus in the dead embryos decreased logarithmically with respect to time, a relationship to be expected if the virus were being destroyed in a first order reaction and no more were being formed. The data indicate an apparent half-life of about 12 hours for the virus of feline pneumonitis in embryos killed by infection and maintained at 37 C after death. A similar rate of virus destruction was also observed in living infected embryos which had been treated with penicillin or aureomycin (Gogolak and Weiss, 1950). It is possible that in both dead and antibiotic-treated embryos the virus has completely ceased to multiply and is simply being destroyed according to first order reaction kinetics. It has been found that purified feline pneumonitis virus, which is also inactivated according to apparent first order kinetics, has a half-life of 90 minutes at 37 C (Moulder and Weiss, 1951a), and the slower decline of infectivity in dead embryos may result from the greater stability of the virus within the host cell.

Examination of histological sections of infected yolk sacs taken from dead chick embryos each time a titration was performed revealed three distinct types of virus activity during the period of decline in virus titer. First, the large, outstretched vesicles typically found in the yolk sacs of living embryos slowly declined in number, and many appeared to have broken up and released elementary bodies into the cytoplasm of the yolk cells. This histological picture is in harmony with the decline in virus concentration indicated by the infectivity titrations. Second, many compact well-rounded clusters and vesicles appeared about 18 hours after death of the embryos and persisted for one or two days thereafter. The rounded appearance of these structures may have been brought about by a decrease in the consistency of the infected cells after death of the embryos. It is not known whether the clusters and vesicles with this abnormal rounded appearance contained infective elementary bodies. Third, many small clusters of normal appearance and containing large granules were seen for several days after death of the embryos. Such structures are usually associated with the actively multiplying virus, and their occurrence in the dead embryos suggests that at least some multiplication of the virus was taking place despite the overall decrease in virus titer.

A tentative picture of the relationship between virus multiplication, host cell metabolism, and death of the embryo may be gained from these studies. The virus titer in the infected embryo rises rapidly without changing the oxygen uptake or the anaerobic glycolysis of the extra-embryonic membranes until the embryo is killed by the infection, and the highest concentration of virus is reached very close to the time of death of the embryo. After the embryo dies, the oxygen uptake of the extra-embryonic membranes falls rapidly, while the anaerobic glycolysis is affected more erratically. The total concentration of infective virus in the dead embryos decreases logarithmically with respect to time, and the vesicles break up and decrease in number, although some virus particles may continue to multiply after death of the embryo.

DISCUSSION

Multiplication of the virus of silk worm jaundice (Gratia et al, 1945) and

of the T2 and T4 viruses of *Escherichia coli* (Cohen and Anderson, 1946) occurs without any increase in the oxygen consumption of the infected cells. Failure of the agent of feline pneumonitis to alter the rate of oxygen uptake and anaerobic glycolysis as it multiplies within the extra-embryonic membranes of chick embryos is in agreement with these observations. That virus reproduction fails to change the overall metabolic rate of infected cells is reasonably explained by the assumption that the virus diverts the enzymes of the host cell from the synthesis of new cellular constituents to the synthesis of new virus without increasing the total energetic requirements of the infected cell (Cohen, 1947). Such observations also lend support to the view that viruses have few, if any, independent enzymes and may be contrasted with the effect of the growth of malarial parasites upon the metabolism of infected erythrocytes. These obligate intracellular parasites possess many powerful enzyme systems of their own, and the growth of malarial parasites is accompanied by a many-fold increase in the oxygen uptake and glycolysis of infected erythrocytes (Moulder, 1948).

Many days after chick embryos have been killed by chilling, the cells of the extra-embryonic membranes remain viable when explanted into tissue culture (Rabinowitz et al, 1948) and exhibit a high rate of oxygen uptake and anaerobic glycolysis. Therefore, the fact that an infectious agent is capable of multiplying in dead embryos is of little help in determining what metabolic processes of the host cell are necessary for reproduction of the agent. Thus, the agents of typhus (Rabinowitz et al, 1948), influenza (Lahelle and Horsfall, 1949), and feline pneumonitis (Weiss, 1950a), which undoubtedly make quite different demands upon the enzyme systems of the infected cell, all are capable of reproducing in the extra-embryonic membranes of dead chick embryos.

In contrast to death of the chick embryo brought about by chilling, death of the embryo caused by infection with feline pneumonitis virus is quickly followed by a rapid decline in the respiratory metabolism of the extra-embryonic membranes. At the same time, virus multiplication stops abruptly, and the virus titer declines along with the respiratory rate. This observation suggests that the agent of feline pneumonitis is strongly dependent upon a high level of host cell metabolism for its reproduction and possesses few if any independent enzyme systems. This belief is in harmony with the previously discussed failure of the virus to influence the respiratory metabolism of infected cells during its reproduction and with the absence of detectable oxygen uptake or glycolysis in purified preparations of the agent (Moulder and Weiss, 1951a).

Since the investigations of Zinsser and Schoenbach (1937) on the physiological conditions prevailing in tissue cultures, it has been generally assumed that "true viruses" multiply most rapidly in cells of high metabolic activity and not at all in dead cells, whereas rickettsiae grow best in cells in which metabolic activity has almost ceased. More recent work has completely justified this conclusion with respect to the rickettsiae. In chick embryos, the rate of multiplication of typhus rickettsiae has been found within reasonable limits to be inversely proportional to the rate of host cell respiration (Greif et al, 1944; Greif and Pinkerton, 1945, 1948), and partially-purified preparations of typhus rickettsiae actively metabolize glutamate and pyruvate (Bovarnick and

Snyder, 1949; Bovarnick and Miller, 1950). Thus, it appears probable that rickettsiae possess a considerable number of independent enzymes which are essential for their reproduction and which function best in host cells of impaired metabolic activity. The dependency of animal and bacterial viruses upon an active host cell metabolism has also been established. The relation between host cell activity and the multiplication of animal viruses has been studied mainly in tissue cultures (reviewed by Enders, 1948). Virus multiplication appears to be greatest when cellular activity is highest, although exceptions to this general statement have been reported. Bacterial viruses also appear to be strongly dependent upon the metabolic activity of the infected bacteria for their reproduction (Hershey and Bronfenbrenner, 1948).

Jones et al (1945) and Rake (1948) have pointed out that the psittacosis-lymphogranuloma group of viruses, of which the agent of feline pneumonitis is a typical member, possess several characteristics which set it apart from the smaller "true viruses" and have suggested that this group of infectious agents, like the rickettsiae, occupies a position intermediate between the bacteria and the smaller viruses. The results of the present study indicate that, in its dependency upon host cell metabolism for its reproduction, the agent of feline pneumonitis more closely resembles the smaller viruses than it does the rickettsiae and the bacteria. It is difficult to compare the results with feline pneumonitis virus with similar information concerning other members of the psittacosis-lymphogranuloma group. Yanamura and Meyer (1941) found that multiplication of psittacosis virus in tissue culture is intimately associated with tissue survival, whereas Sanders (1940) observed that the agent of lymphogranuloma venerum multiplies best in tissue cultures held at 23 C, well below the optimum temperature for host cell growth and respiration.

SUMMARY

Consistent values for the oxygen uptake ($Q_{O_2} = 5.6$) and the anaerobic glycolysis ($Q^{N_2}_{CO_2} = 5.0$) of the extra-embryonic membranes of chick embryos were obtained when the fat-free dry weights of the tissues were used in the calculations. The oxygen uptake and anaerobic glycolysis of extra-embryonic membranes from chick embryos 5 to 13 days of age showed no apparent variation with respect to age of embryo.

Infection of chick embryos with the agent of feline pneumonitis did not change the Q_{O_2} or $Q^{N_2}_{CO_2}$ of extra-embryonic membranes taken from living, infected embryos.

When chick embryos were killed by chilling and then reincubated at 37 C, the oxygen consumption and anaerobic glycolysis of the extra-embryonic membranes were first higher than in living embryos, slowly declined to the level of living embryos within 4 to 7 days, and at 12 days after death were about 50% of the values obtained in living embryos. Growth of the agent of feline pneumonitis in dead embryos produced no change in the respiratory metabolism of the extra-embryonic membranes.

When chick embryos were killed by infection with the agent of feline pneumonitis, the Q_{O_2} of the infected membranes declined to a low level within 24 hours after death of the embryos. At the same time virus multiplication ceased, and the virus titer declined along with the respiratory rate.

These studies suggest that the agent of feline pneumonitis is dependent

upon a high level of host cell metabolism for its multiplication.

REFERENCES

Bovarnick, M. R. and Miller, J. C. 1950, J. Biol. Chem. **184:** 661–679.

Bovarnick, M. R. and Snyder, J. C. 1949, J. Exper. Med. **89:** 561–565.

Bucciante, L. 1931, Arch. f. exper. Zellforsch. **11:** 397–423.

Cohen, S. S. 1947, Cold Spring Harbor Symp. Quant. Biol. **12:** 35–49.

Cohen, S. S. and Anderson, T. F. 1946, J. Exper. Med. **84:** 511–523.

Dixon, M. 1943, Manometric methods, ed. 2. New York, Macmillan, pp. 49–68.

Enders, J. F. 1948, Viral and Rickettsial Infections of Man, T. M. Rivers, ed. Philadelphia, Lippincott, pp. 114–127.

Gogolak, F. M. and Weiss, E. 1950, J. Infect. Dis. **87:** 264–274.

Gratia, A., Brachet, J. and Jeener, R. 1945, Bull. Acad. roy de méd. de Belgique **10:** 72–81.

Greif, D., Pinkerton, H. and Moragues, V. 1944, J. Exper. Med. **80:** 264–274.

Greif, D. and Pinkerton, H. 1945, J. Exper. Med. **82:** 193–206.

———. 1948, J. Exper. Med. **87:** 175–197.

Hershey, A. D. and Bronfenbrenner, J. 1948, Viral and Rickettsial Infections of Man, T. M. Rivers, ed. Philadelphia, Lippincott, pp. 147–164.

Jones, H., Rake, G. and Stearns, B. 1945, J. Infect. Dis. **76:** 55–69.

Krebs, H. A. and Henseleit, K. 1932, Zeit. physiol. Chem. **210:** 33–66.

Lahelle, O. and Horsfall, F. L., Jr. 1949, Proc. Soc. Exper. Biol. & Med. **70:** 547–551.

Moulder, J. W. 1948, Annual Review Microbiol. **2:** 101–120.

Moulder, J. W. and Weiss, E. 1951a, J. Infect. Dis. **88:** 56–67.

———. 1951b, J. Infect. Dis. **88:** 77–80.

Rabinowitz, E., Aschner M. and Grossowics, N. 1948, Proc. Soc. Exper. Biol. & Med. **67:** 469–470.

Rake, G. 1948, In Bergey's Manual of Determinative Bacteriology, R. S. Breed, E. G. D. Murray, and A. P. Hitchens, ed. 6. Baltimore, Williams and Wilkins.

Sanders, M. 1940, J. Exper. Med. **71:** 113–128.

Weiss, E. 1950a, J. Infect. Dis. **86:** 27–32.

———. 1950b, J. Infect. Dis. **87:** 249–263.

Weiss, E., Moulder, J. W. and Itatani, M. K. 1951, J. Infect. Dis. In press.

Yanamura, H. Y. and Meyer, K. F. 1941, J. Infect. Dis. **68:** 1–15.

Zinsser, H. and Schoenbach, E. B. 1937, J. Exper. Med. **66:** 207–227.

THE EFFECT OF MURINE PNEUMONITIS AND LIPID PNEUMONIA UPON THE METABOLISM OF MOUSE LUNG

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As described in the preceding paper (Moulder and Weiss, 1951) the rates of oxygen consumption and anaerobic glycolysis of the extra-embryonic membranes of chick embryos infected with the agent of feline pneumonitis are the same as in uninfected controls. In contrast, infection of mice with murine pneumonitis virus produces a sharp reduction in the oxygen uptake and a smaller but definite rise in anaerobic glycolysis of the infected lungs. However, for reasons to be described herein, it is probable that these metabolic changes are due, not to the reproduction of the virus, but instead, to the inflammatory response of the infected tissue.

MATERIALS AND METHODS

Inoculation of mice.—The Chicago strain of murine pneumonitis was the same as used in previous work in this laboratory (Weiss, 1950). For preparation of virus pools for inoculation of mice, heavily infected lungs were ground in a mortar and pestle with powdered glass and sufficient veal infusion broth to give a 10% emulsion. The ground material was centrifuged briefly to remove large tissue particles, and the supernatant liquid was divided into several parts and stored in the dry ice box until used. Lipid pneumonia was produced by the intranasal instillation of sterile egg yolk. Swiss mice of 7 to 15 g weight were inoculated by the intranasal instillation of 0.03 ml of either the virus pool or the sterile egg yolk, while under light ether anesthesia. The virus was administered in varying concentrations, depending upon the degree of consolidation desired. The mice were killed by a blow on the head 1 to 7 days after inoculation. and the lungs were removed with aseptic precautions. The lesions were classified on the basis of the degree of consolidation of the pulmonary tissue and divided into four classes: one-fourth, one-half, three-fourths, and complete consolidation.

Manometric techniques.—As reported by Boyland and McDonald (1948), the intact lungs of young mice are sufficiently small to allow measurement of gas exchange in the Barcroft-Warburg apparatus without slicing or mincing. Oxygen consumption, expressed as Q_{O_2},† and anaerobic glycolysis, expressed as $Q^{N_2}_{CO_2}$,‡ were determined for the lungs of each mouse studied. The left lung of each mouse was routinely used for oxygen uptake, and the right lung was used for anaerobic glycolysis. Reversal of this procedure did not alter the results. Each lung was blotted on filter paper, weighed to the nearest mg on a torsion balance, and oxygen consumption and carbon dioxide evolution were measured under precisely the same conditions as described in the preceding paper (Moulder and Weiss, 1951). At the end of an hour in the Barcroft-Warburg apparatus, the lungs were removed from the flasks. One lung from each animal was smeared on blood agar plates to determine the extent of bacterial contamination. Most lungs showed the presence of only insignificant numbers of bacteria, and the few heavily contaminated samples were discarded. The other lung from each animal was dried at 105 C for two hours. This dry weight

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† Q_{O_2} = microliters of oxygen consumed in 1 hour by 1 mg dry tissue.

‡ $Q^{N_2}_{CO_2}$ = microliters of carbon dioxide liberated under anaerobic conditions from bicarbonate buffered medium in 1 hour by 1 mg dry tissue. In the tissues of higher animals, this value is approximately equal to the amount of lactic acid formed from glucose, and is, thus, a measure of the rate of anaerobic glycolysis.

was used in calculation of the Q values for both lungs of a given mouse. This procedure appeared justified because the dry weights of the mouse lungs were not affected by the degree of consolidation. The mean dry weight/wet weight ratios for the three groups of experimental animals were: 0.172 ± 0.020 for the uninoculated controls, 0.158 ± 0.018 for the animals inoculated with murine pneumonitis virus, and 0.165 ± 0.021 for the mice inoculated with sterile egg yolk.

metabolism of mouse lung.* The oxygen consumption and anaerobic glycolysis of infected lungs have been plotted against the degree of consolidation of the pulmonary tissue, because the degree of consolidation is a convenient index of the extent and intensity of the infection. The inhibition of oxygen con-

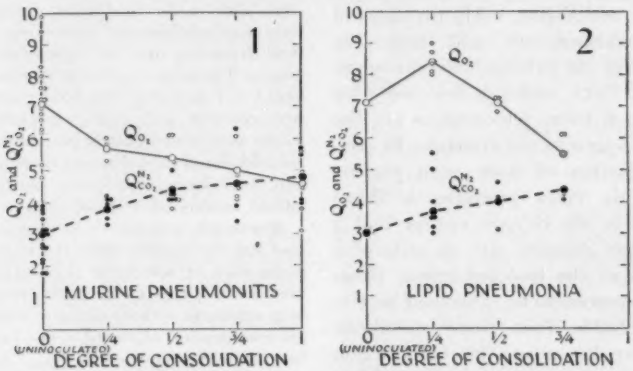

FIG. 1.—Effect of infection with the agent of murine pneumonitis upon the metabolism of mouse lung. Individual observations are indicated by small circles, means by large circles. Q_{O_2} is represented by open circles, and the means are connected by a continuous line. $Q^{N_2}_{CO_2}$ is shown by solid circles, and the means are connected by a broken line.

FIG. 2.—Effect of lipid pneumonia upon the metabolism of mouse lung. The notation is the same as in figure 1. The same group of animals served as the uninoculated controls, and only the means of this group are shown here.

EXPERIMENTAL RESULTS

Respiratory metabolism of normal mouse lung.—The oxygen uptake (Q_{O_2}) and anaerobic glycolysis ($Q^{N_2}_{CO_2}$) of 14 normal uninoculated mice is recorded in figure 1 as the values for zero consolidation. The mean Q_{O_2}, 7.1, agrees well with the mean of 7.3 reported by Boyland and McDonald (1948). The mean $Q^{N_2}_{CO_2}$, 3.0, is the same as that reported by these workers.

Effect of infection with the agent of murine pneumonitis upon the respiratory metabolism of mouse lung.—Figure 1 shows the effect of infection with the agent of murine pneumonitis upon the

sumption and stimulation of anaerobic glycolysis is apparent even in light infections with little consolidation and becomes more marked with increasing consolidation. In fully consolidated lungs, the ratio of $Q_{O_2}/Q^{N_2}_{CO_2}$ is less than half that of unconsolidated lungs. Infected lungs show a generalized decrease in oxidative capacity, for the decrease in oxygen uptake also occurs when pyruvate, succinate, glutamate, or *p*-phenylenediamine are substituted for

* Infection with the agent of feline pneumonitis produces entirely similar changes in the respiratory metabolism of mouse lung.

glucose as the substrate. By dissecting partially consolidated lungs into consolidated and unconsolidated portions, it has been found that the respiratory changes are localized almost entirely in the consolidated portions. Since the agent of murine pneumonitis gives immediate toxic reactions when injected into mice (Rake and Jones, 1944), extracts of normal and heavily infected lungs were tested for their effect upon the rate of oxygen uptake and anaerobic glycolysis in intact normal lungs. However, none of these extracts exerted any observable metabolic inhibition.

Effect of lipid pneumonia upon the respiratory metabolism of mouse lung.— Since the respiratory changes occurring in infections with murine pneumonitis virus seemed to be so intimately related to the consolidation of the lung tissue, the effect of pulmonary consolidation produced by a non-multiplying agent was next investigated. As shown in figure 2, the changes in oxygen consumption and anaerobic glycolysis accompanying the consolidation of mouse lung produced by lipid pneumonia are very similar to those produced in murine pneumonitis. Lung consolidation produced by inoculation of sterile egg yolk was less extensive than in murine pneumonitis, and complete consolidation was never observed. However, extrapolation of the mean curves for Q_{O_2} and $Q_{CO_2}^{N_2}$ in lipid pneumonia to complete consolidation gives approximately the same values obtained in fully consolidated lungs from mice infected with the agent of murine pneumonitis. The early stages of lipid pneumonia are accompanied by a small rise in oxygen uptake which cannot be explained from the present data.

DISCUSSION

The decrease in oxygen uptake and increase in anaerobic glycolysis observed in lungs from mice infected with the agent of murine pneumonitis might conceivably result from (1) the metabolism of the virus itself, (2) synthetic reactions carried out by the host but closely associated with reproduction of the virus, or (3) the inflammatory response of the infected tissue. The fact that similar metabolic changes do not occur in the extra-embryonic membranes of chick embryos infected with the closely related agent of feline pneumonitis (Moulder and Weiss, 1951) makes the first two explanations unlikely, because such alterations in metabolism should be associated with the proliferation of the virus in any tissue. The third explanation, which ascribes the observed changes in respiratory metabolism to the inflammatory processes occurring in infected lung, appears to be the most plausible at present and is supported by the similarity of effect produced by the infectious agent and by egg yolk. The lack of appreciable inflammation in virus-infected yolk sac would explain the lack of respiratory alterations in this tissue.

If the inflammatory response of the lung is indeed responsible for the changes in oxygen uptake and anaerobic glycolysis, then mouse lung is well suited for a biochemical study of inflammation. The lung is small enough to be used for manometric determinations without slicing or mincing, and the alveolar structure of the lung allows a very large cellular infiltration. The infiltrated cells may have a metabolic pattern different from that of the cells originally present in the normal lung, but it is difficult to correlate the type of cell predominant in a given inflammatory reaction with the type of respiratory changes which occur. The response in the early stages of murine pneumonitis is probably chiefly heterophil in nature; lipid pneumonia is accompanied by a predominantly lymphocytic infil-

tration; yet both of these conditions bring about similar changes in Q_{O_2} and $Q_{CO_2}^{N_2}$. On the other hand, the inflammatory-like changes in chicken spleen during infection with *Plasmodium gallinaceum*, which are mainly lymphoid in nature, cause no observable change in the oxygen consumption or anaerobic glycolysis of spleen slices (Taliaferro and Moulder, unpublished results). It is doubtful if the consolidation of the lung tissues reduces oxygen consumption by interfering with the diffusion of oxygen through the consolidated areas, because normal mouse lung, which collapses completely within 30 minutes after being incubated in the Warburg apparatus at 37 C, continues to consume oxygen at a constant rate for as long as two hours.

Because the respiratory changes described here can be attributed to inflammation and not to direct action of the virus, the effect of the agent of murine pneumonitis upon the metabolism of mouse lung remains obscure.

SUMMARY

Lungs from mice infected with the agent of murine pneumonitis exhibit a decreased rate of oxygen consumption and an increased rate of anaerobic glycolysis. These changes in metabolism of mouse lung are proportional to the degree of consolidation of the pulmonary tissue. Lipid pneumonia produced in mice by the intranasal instillation of egg yolk produces respiratory changes similar to those occurring in murine pneumonitis. It is suggested that the metabolic changes occurring in mouse lungs infected with murine pneumonitis virus are due to the inflammatory response of the lung, and not to growth of the virus.

REFERENCES

Boyland, E. and McDonald, F. F. 1948, Biochem. J. **42**: 68–71.

Moulder, J. W. and Weiss, E. 1951, J. Infect. Dis. **88**: 68–76.

Rake, G. and Jones, H. P. 1944, J. Exper. Med. **79**: 463–486.

Weiss, E. 1950. J. Infect. Dis. **87**: 249–263.

THE INHIBITORY ACTION OF SALIVA ON THE DIPHTHERIA BACILLUS: HYDROGEN PEROXIDE, THE INHIBITORY AGENT PRODUCED BY SALIVARY STREPTOCOCCI

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In a previous report (Thompson and Shibuya, 1946) it was shown that the inhibitory action of saliva on the diphtheria bacillus was due to the mitis type viridans streptococci (Sherman et al, 1943) present in the saliva. It was also shown that certain other salivary organisms (particularly staphylococci) antagonized the inhibitory action. In view of the production of hydrogen peroxide by many streptococci (McLeod and Gordon, 1922, 1923, and others), and of catalase by staphylococci (McLeod and Gordon, 1923), it was possible that the inhibitory action of salivary streptococci was due to hydrogen peroxide,* and that the antagonism produced by staphylococci was due to catalase.

METHODS

Except where otherwise noted, all tests for inhibition were made by placing standard drops of the materials to be tested on the surfaces of agar pour plates containing various concentrations of diphtheria bacilli. The zones of growth inhibition around the drops were measured after suitable incubation. Details of the method, description of the medium and of the diphtheria bacillus strain used in the tests were given in the previous report (Thompson and Shibuya, 1946).

TABLE 1.—*Correlation of inhibitory action of salivary organisms with the production of hydrogen peroxide.*

Type of organism	Number of strains tested	Results of test for inhibition	Number in each group which produced hydrogen peroxide
Streptococci showing green zones on blood agar	76	69+ 7−	69 0
Streptococci indifferent on blood agar and producing mucoid colonies on 5% sucrose*	10	0+ 10−	0 0
Staphylococci	34	0+ 34−	0 0
Diphtheroids	10	1+ 9−	0 0

* Salivarius type of Sherman et al, 1943.

Hydrogen peroxide production was detected in several ways. (1) Sixty milligram percent of benzidine were added to the following medium while still hot after sterilization: Tryptose, 2%; sodium chloride, 0.5%; glucose, 0.05%; agar, 1.5%. Juice pressed from potatoes was filtered through a Seitz filter and added to make a 2% concentration after the medium had cooled to 45 C. Organisms to be tested for peroxide production were inoculated onto the surfaces of plates of this medium, which were then incubated for 24 hours. A brown color of varying intensity in and around the colonies was regarded as an indication of peroxide production. Hydrogen peroxide solutions dropped onto such plates produced a blue color which changed to brown on standing. (2) To broth cultures, or filtrates of

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* Recently, Arvidson and his co-workers (1950) reported that streptococci from the salivary and respiratory tract inhibit diphtheria bacilli in certain mediums. They found the inhibitory action to be antagonized by red blood cells, but did not consider the possibility that hydrogen peroxide might be concerned. Dr. C. B. Van Niel suggested to us this possibility.

these, were added a small cube of fresh potato and one-tenth volume of a saturated glacial acetic acid solution of benzidine (Avery and Morgan, 1924). A blue color developing on the surface of the potato indicated peroxide production. (3) For quantitative estimations of peroxide, the method of Main and Shinn (1939) was used. To 1 ml of broth culture or filtrate thereof, 6 drops of centrifuged and filtered potato juice, diluted 1:4 with saline, were added. The intensity of the blue color which developed was compared with that of Malachite green standards which, in turn, had been standardized against hydrogen peroxide solutions of known concentrations.

Catalase production was detected by pouring 3 ml of a 3% solution of hydrogen peroxide over 24 hour agar slant cultures, or by adding 1 ml of the solution to Smith's fermentation tube cultures containing 30 ml of broth culture. Formation of gas bubbles was regarded as an indication of catalase action.

Correlation of inhibitory action with the production of hydrogen peroxide.—Strains of various bacteria isolated from the salivas of 12 individuals were tested for inhibitory action on the diphtheria bacillus (stock strain) by the plate method, and for hydrogen peroxide production by one or more of the methods described above. The results are summarized in table 1.

TABLE 2.—*Inhibitory power of streptococcal strains compared with hydrogen peroxide concentrations produced.*

Experiment	Streptococcus no.	Concentration of hydrogen peroxide by rocked culture (μg/ml)	Diameters of zones of inhibition on plates (mm)
1	1	22	17.5
	2	20	10
	3	16	7
2	4	22	20
	5	20	13
	6	20	13
	7	20	12
	8	20	12
	9	20	11
	10	16	9
	11	16	9
	12	16	8
	13	0	0
			Highest dilution of culture filtrate inhibiting growth of diphtheria bacillus
3	4	20	1:8
	9	20	1:6
	11	12	1:4
	13	0	0

A number of strains of streptococci were inoculated into shallow layers of broth (2% tryptose) in flasks which were mechanically rocked during incubation. The hydrogen peroxide concentrations in these rocked cultures were roughly estimated by the benzidine-potato cube method; the intensity of the blue color on the potato surface indicating the relative concentration of hydrogen peroxide produced. The strains producing the most intense blue color were invariably the best inhibitors on the pour plates. The hydrogen peroxide producing capacity of a limited number of the streptococci was tested by the more accurate titration method described above. The inhibitory powers were determined either by the plate method or by the inoculation of the diphtheria bacilli into dilutions of filtrates of the rocked cultures. There was a close correlation between the concentration of peroxide produced by a strain and its ability to inhibit the diphtheria bacillus. The results of three such experiments are given in table 2.

Catalase solutions were added to several of the cultures showing the g eatest concentrations of peroxide. Definite formation of gas bubbles indicated that the peroxide giving the color reaction actually was hydrogen peroxide.

Attempts were made to detect the possible production, by the streptococci, of another inhibitory agent in addition to the peroxide. Five strains of active streptococci were grown in deep flasks of broth with relatively small surfaces exposed to the air. After 24-hour growth, no peroxide could be detected in any of these cultures, and the stock diphtheria bacillus grew well in undiluted filtrates of these cultures. Portions of the same cultures were transferred to larger flasks and aerated by shaking for 10 to 15 minutes. Concentrations of hydrogen peroxide from 8 to 24 μg per ml were obtained, and filtrates of the aerated cultures inhibited the diphtheria bacillus when diluted from 4 to 8 times. The streptococci did not produce any inhibitory agent when grown under conditions preventing formation of hydrogen peroxide.

Production of catalase by organisms antagonizing the inhibitory action of salivary streptococci

Various organisms isolated from saliva were studied as to their ability to produce catalase and to antagonize the inhibitory action of saliva and of salivary streptococci. Antagonism was detected by placing a heavy inoculum of the organism to be tested a short distance from the saliva or streptococcus drops on the diphtheria pour plates. After incubation the zones of inhibition produced by the salivary streptococci were measured both in the vicinity of, and away from the inoculum being tested. A narrowing of the zone of inhibition in the vicinity of the inoculum

was interpreted as antagonism of the inhibitory agent (fig. 1). Methods for testing for catalase production were given above. Table 3 summarizes the results obtained.

TABLE 3.—*Correlation of catalase production and antagonism of inhibition.*

Type of organism	Number of strains tested		Number of strains showing antagonism	Number of strains producing catalase
Staphylococci	29	29 against streptococcal cultures	29	29
		7 against saliva*	7	
Diphtheroids	9	9 against streptococci	9†	9
		9 against saliva	9	
Salivarius type streptococci	10	10 against streptococci	0	0
		10 against saliva	0	
Mitis type streptococci	19	19 against streptococci	0	0

* Several specimens of saliva and several strains of inhibitory streptococci were used in each test.

† An error was made in one sentence of our previous report which incorrectly stated that diphtheroids did not antagonize (Thompson and Shibuya, 1946).

There was an absolute correlation between the ability of an organism to antagonize the inhibitory action of saliva or of pure cultures of salivary streptococci and its ability to produce detectable catalase. Potato juice and lysed blood cells both antagonized the inhibitory actions of saliva and streptococci. Saliva containing few catalase producing bacteria produced more active inhibition than saliva containing many catalase producers. The evidence clearly indicated that the antagonism of the inhibitory action of salivary streptococci by the other salivary bacteria was due to catalase production. It also strengthened the hypothesis that the inhibitory activity of the streptococci results from the production of hydrogen peroxide.

Comparison of susceptibilities of various organisms to the inhibitory actions of saliva, salivary streptococci and hydrogen peroxide

Various organisms as indicated in table 4 were tested for susceptibility to inhibition by 2 specimens of saliva, 2 strains of salivary streptococci and by hydrogen peroxide (0.1%). The pour plate method, as used throughout this work for the diphtheria bacillus, was used. The peroxide solutions were dropped onto filter paper disks placed on the agar surfaces, The results of seven separate experiments are summarized in table 4.

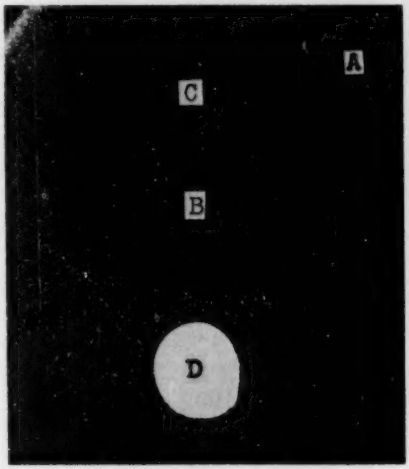

FIG. 1.—Staphylococcal Antagonism of Streptococcal Inhibition.
A. Growth of Diphtheria Bacilli.
B. Growth of Mitis Streptococci.
C. Zone of Inhibition.
D. Colony of Staphylococci.

Within the limits of the error involved in the various procedures, there was a close relationship between the susceptibilities of the organisms to the three agents. Unless an organism was markedly susceptible to hydrogen peroxide (zones of inhibition of more than 10 mm), it was not appreciably affected by saliva or streptococci. The diphtheria bacilli, the white staphylococcus and the salivarius type streptococcus were most susceptible to the three agents. The gram negative bacilli were

least susceptible. These results were consistent with the hypothesis that the inhibitory action of salivary streptococci and of saliva is due to hydrogen peroxide.

The effect of changes in tryptose concentration in the medium on the inhibitory action of hydrogen peroxide

In the previous report it was shown that the inhibitory actions of both saliva and salivary streptococci were best demonstrated in media with reduced tryptose content; being completely abolished with the standard 2% tryptose. The inhibitory action of hydrogen peroxide solutions was likewise diminished by increasing the tryptose content of the medium. The effect was very definite, but not as striking as with saliva or streptococci.

DISCUSSION

The inhibitory action of saliva on the diphtheria bacillus would appear from these experiments to be largely due to the hydrogen peroxide produced by mitis type viridans streptococci. Any pneumococci present would undoubtedly play a similar role, since they are known to produce hydrogen peroxide. We have found that several types of pneumococci from other sources inhibited the diphtheria bacilli just as did the streptococci, but we have not included them in this paper because, possibly for technical reasons, we did not isolate pneumococci from any of the specimens of saliva used. It is probable that occasionally the respiratory membranes may harbor organisms which produce other types of antibiotic agents. We obtained from saliva one diphtheroid

TABLE 4.—*Inhibition of various organisms by saliva, streptococci, and hydrogen peroxide.*

Experiment	Organism	Maximum zones of inhibition in mm produced by:				
		Saliva		Streptococcus		Hydrogen peroxide (0.1%)
		Nos. 1*	2	1	2	
1	*Shigella shigae*	0	0	0	+	5
	Shigella sonnei	0	0	0	1	6
	Corynebacterium diphtheriae #6	6	11	5	15	15
2	*Corynebacterium diphtheriae* #40	3	12	5	13	18
	Corynebacterium diphtheriae #X	4	12	6	12	18
	Corynebacterium diphtheriae #6	4	11	6	13	19
3	*Staphylococcus aureus*	+	2	6	9	12
	Staphylococcus albus	8	8	11	13	14
	Corynebacterium diphtheriae #6	12	14	13	18	14
4	*Escherichia coli*	0	0	0	0	5
	Salmonella typhi	0	0	0	0	4
	Salmonella paratyphi	0	+	0	0	4
	Corynebacterium diphtheriae #6	5	13	13	13	13
5	Diphtheroid #1	2	4	4	5	14
	Diphtheroid #2	0	0	0	0	8
	Diphtheroid #3	0	0	0	0	5
	Diphtheroid #4	0	+	0	0	6
	Streptococcus salivarius	7	11	11	7	12
	Corynebacterium diphtheriae #6	8	9	11	11	23
6	*Corynebacterium diphtheriae mitis*	2	11	15	13	14
	Corynebacterium diphtheriae #6	1	6	10	8	10
7	Aerobic actinomycete #1†	+	2	3	4	11
	Aerobic actinomycete #2	1	1½	3	5	10
	Corynebacterium diphtheriae #6	4	5	7½	10	12

+ = Inhibition under drop but no zone around it.
* Saliva specimens in the different experiments were not from the same individuals.
† Gram positive branching organisms isolated from saliva.

strain which inhibited the diphtheria bacillus but which produced no peroxide. Staphylococci which inhibit diphtheria bacilli have been described (Dujardin-Beaumetz, 1932; Duliscouet, 1935) and Tong, in this laboratory, has shown that two strains of coagulase positive staphylococci, isolated from infections, markedly inhibited our test diphtheria bacillus. These staphylococci produced catalase but not hydrogen peroxide. The occasional occurrence in saliva of organisms inhibiting the diphtheria bacillus by other mechanisms does not alter the fact that the inhibitory properties of most salivas are due to the hydrogen peroxide produced by the mitis type streptococci.

The relationship of catalase production to the antagonism of the inhibitory property by other salivary organisms strengthens the case for hydrogen peroxide as the inhibitory agent, as does the antagonism by such catalase-containing materials as potato and blood. The presence of such catalase producing, antagonizing organisms in varying numbers in different salivas raises the question as to whether the relative ratio of peroxide producers to catalase producers on certain respiratory membranes may play a role in susceptibility or resistance to some infections. Viruses are recognized to be extremely sensitive to hydrogen peroxide.

SUMMARY

One hundred and thirty strains of salivary bacteria were compared as to their power to inhibit the growth of the diphtheria bacillus and to produce hydrogen peroxide. With one exception (a diphtheroid bacillus) no organism inhibited unless it produced hydrogen peroxide. All the strains which produced hydrogen peroxide and inhibition were mitis type viridans streptococci. The inhibitory capacity of these organisms was proportional to the concentration of peroxide which they were able to produce.

Sixty-seven strains of salivary bacteria were compared as to their ability to antagonize the inhibitory action of saliva and of salivary streptococci and to produce catalase. Thirty-eight strains (staphylococci and diphtheroid bacilli) both antagonized the inhibitory action and produced catalase. Twenty-nine strains (mitis and salivarius type streptococci) neither antagonized nor produced catalase.

Seventeen strains of various bacteria were compared as to their susceptibility to inhibition by saliva, salivary streptococci and hydrogen peroxide. The order of sensitivity was the same to all three agents.

REFERENCES

Arvidson, M., Ericsson, H., Ouchterlony, O. and Vahlquist, B. 1950, Acta path. et microbiol. Scandinav. **27**: 263–269.

Avery, O. T. and Morgan, H. J. 1924, J. Exper. Med., **39**: 275–287.

Dujardin-Beaumetz, E. 1932, Compt. rend. Soc. de biol. **110**: 1210–1213.

Duliscouet, R. 1935, Compt. rend. Soc. de biol. **118**: 1277–1280.

Main, E. R. and Shinn, L. E. 1939, J. Biol. Chem. **128**: 417–423.

McLeod, J. W. and Gordon, J. 1922, Biochem. J. **16**: 499–506.

———. 1923, J. Path. & Bact. **26**: 326–331.

Sherman, J. M., Niven, C. F., Jr. and Smiley, K. L. 1943, J. Bact. **45**: 249–263.

Thompson, R. and Shibuya, M. 1946, J. Bact. **51**: 671–684.

Tong, J. L. Personal communication.

Since this paper was sent to press, we have received the report of Hegemann (Zeit. f. Hygiene, 1950, 131, 355–363) on an antibacterial agent produced by green producing salivary streptococci, in which he used *Staphylococcus aureus* as the test organism. He concluded that the active agent was "probably" peroxide because it was inhibited by fresh blood, was not produced anaerobically, and had strong oxidizing properties.

THE ADAPTATION OF THE AVIAN MALARIA PARASITE *PLASMODIUM LOPHURAE* TO A CONTINUOUS EXISTENCE IN INFANT MICE

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The erythrocytes of the rabbit, pig, mouse, and, to a lesser extent, man are susceptible to infection with the avian malaria parasite *Plasmodium lophurae* when these erythrocytes are present in the blood stream of infected chick embryos.[1,2] The infant white mouse is also subject to a transient infection after intravenous inoculation from an infected chick embryo.[3] This paper will report in detail on initial infections of mice and will relate the findings observed during adaptive passages which ultimately resulted in the establishment of a murine strain of *P. lophurae*.

MATERIALS AND METHODS

The 12A strain of *P. lophurae*, which was isolated by Coggeshall[4] from the fireback pheasant *Lophura ignili ignili* in the New York Zoological Park, was used. It is important to note that although the host bird is a native of Borneo, the exact origin of the infection is unknown. Infections have been carried separately through Pekin ducks and Rhode Island Red chickens in this laboratory. In 1948, infections from chickens were successfully maintained in White Leghorn chick embryos.[5] Preliminary mouse infections were initiated from this embryo passage. More recently chick embryos were infected with parasites from infected ducklings, and it was from these infections that the data to be presented were drawn. The Swiss strain of the albino mouse was chosen as the experimental host because of its ready availability, its small size (thus requiring a smaller inoculum), and its copious supply of young.

Embryo infections were maintained by intravenous passages at 4-day intervals, and the course of infection was followed by daily blood films. Adaptive passages were begun in embryos as near 10 days of age as possible and were maintained separate from the stock embryo infections. When parasitemias had reached a suitable level (70 to 100 parasites per 100 cells), blood was withdrawn from one of the large chorioallantoic veins into a solution of 27 mg percent heparin in sodium chloride at a proportion of 0.1 ml per 1 ml of blood. The blood suspension was inoculated directly without washing. Adult mice were given injections into a tail vein in the usual manner. Infant mice were placed ventrad upon absorbent paper and the tail quickly secured with cellulose tape. A 28 or 30 gauge needle was inserted through the tape into a tail vein and the blood slowly introduced. As much as 0.15 ml of infected blood has been injected into infant mice in this manner without apparent injury to the recipient. Immediately after inoculation one of the foot pads was pricked with a sharp needle and sufficient blood to make a blood film was drawn into a capillary pipette. Subsequent blood films were made by snipping off a bit of the tail and expressing a drop of blood on a glass slide. Excessive bleeding was rare, and when it did occur the mouse was discarded.

The injection of mouse blood into the chick embryo necessitated washing, since whole blood of even infant mice quickly brought about the death of the embryo. Mice were anesthetized with ether, and the brachial artery was exposed and severed. The blood which collected in the pouch formed by the skin and body wall was aspirated and exhausted into a tube containing a solution of heparin. About 0.1 ml of blood from each mouse could be secured in this manner and it was therefore necessary to pool the blood of several mice to ensure enough material for successful

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1. McGhee, R. B. 1949, Proc. Soc. Exper. Biol. & Med. **71**: 92–93.
2. McGhee, R. B. 1950, Am. J. Hyg. In press.
3. McGhee, R. B. 1949, J. Parasitol. **35**: Sect. 2: 23.
4. Coggeshall, L. T. 1938, Am. J. Hyg. **27**: 615–618.
5. McGhee, R. B. 1949, J. Parasitol. **35**: 411–416.

transfers. After two washings in buffered saline the suspension was made up to its original volume and injected into one of the chorioallantoic veins of the embryo. Blood for mouse-to-mouse passages was secured in the same way but was not washed.

All blood films were stained with Giemsa's stain. Parasites were counted in relation to the erythrocytes, and enough parasites were counted to reduce the probable error to 15 or 20%.

EXPERIMENTAL

Course of infection

Ten-day chick embryos were infected by intravenous inoculation of blood from a heavily infected duckling. After 4 passages in embryos, infection had reached a generally predictable course, and 0.1 ml. of heavily parasitized blood was given intravenously to mice of less than a day of age. Immediately after inoculation blood films were made which revealed a high proportion of avian cells, reaching at times 45% of the total (fig. 1, A1). The introduced erythrocytes were either partially or completely removed on the day following inoculation. If completely removed, the number of mouse cells infected was significantly higher than when only partial clearance of cells was effected. In A1, figure 1, the percent of avian to mouse cells after 24 hours was 33, and the mouse cell infection was 70 parasites per 10,000 erythrocytes; but in A2, in which mouse the bird erythrocytes were reduced from 40% to 4% in the 24-hour period, the initial infection was 180, followed on the 2nd day by a slight rise in numbers. In A3 all avian cells were removed, and the mouse cell invasion reached the greatest peak attained in that infection. Upon further examination it was discovered that the parasitemia in the avian cells had increased in certain mice during the 24-hour period. In A1 the avian cell parasitemia had increased from 6500 to 8400 parasites per 10,000 avian erythrocytes. There was no such increase when most of the avian cells had been removed (fig. 1, A2).

It was not possible, because of the small size of the host, to procure sufficient blood films in a 24-hour period to ascertain the asexual cycle. However, after 40 hours very young trophozoites and segmenting forms were seen which would place the period of maximum segmentation just prior to this time. A differential count of 50 parasites was made on all mice and indicated a synchronism more marked than that generally ascribed for *P. lophurae* in the chicken (fig. 1). As the infection progressed the proportion of ring forms was reduced, indicating less synchronism of reproduction. In no mouse of this group was a peak attained which equaled that of the initial invasion, and the course of infection was characterized by a steady decrease in parasite numbers although sometimes slight declining peaks occurred. In the majority of animals it was impossible to find parasites after the 8th day.

Since these parasites retained their ability to infect chick embryo cells, it was decided to attempt to adapt the parasite to the mouse by alternate embryo-mouse infections. Accordingly, at a time when it was just possible to find parasites in the mouse, all the mice were killed, and the blood was pooled, washed, and passed back to the avian embryo. Two and sometimes three passages through chick embryos were required to produce a parasitemia of such proportions suitable for transfer to the mouse. These embryo passages and the subsequent mouse infection were regarded as one adaptive passage. Four different groups of mice were inoculated, but only one series gave promise enough to warrant continued attempts at adaptation. Only this sequence of passages is figured (fig. 2), and even in this series many passages were attempted and dis-

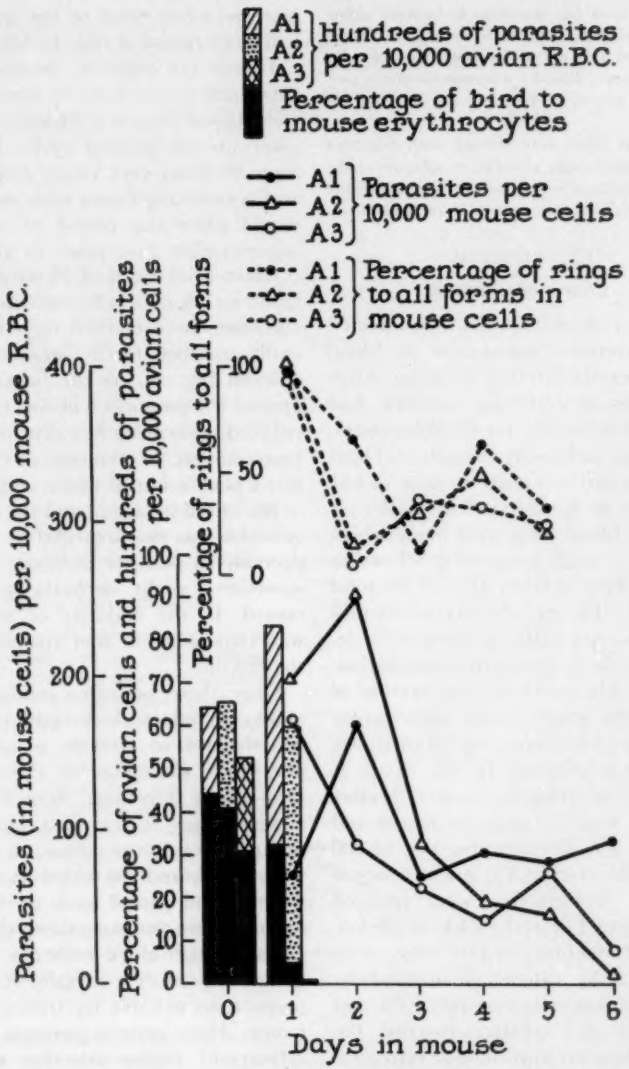

FIG. 1.—The course of infection of *P. lophurae* in initial infections in infant mice and the relation of the rate of removal of avian cells to mouse cell parasitemia.

carded either because of a reduced ability to infect or a lack of improvement over another passage.

The initial passage has been described and is further represented by figure 3 A. The 1st passage (fig. 3 B) was in many respects similar to the initial passage, but the peaks arising after the initial

parasitemia were more marked and in one mouse actually exceeded the initial peak. Moreover, the infection remained patent for a greater period, and, as a result, the transfer back to the chick embryo was not made until 10 days after infection. The 2nd passage was also similar, but the parasitemia did not approach the secondary peak levels seen in the 1st passage. Again, all animals were killed on the 8th day after inoculation. A definite change was seen in the 3rd passage (fig. 3 D) in which the secondary peak, which arose on the 5th day, was significantly higher than that of the initial invasion. The decline of parasite numbers was more gradual and parasites could be detected through 12 days. The 4th passage (fig. 3 E) was similar to the 3rd, but in one mouse the peak parasitemia was 490 parasites per 10,000 erythrocytes. In addition there was another peak reached on the 7th day and a slight relapse in 2 mice occurring on the 10th or 11th day.

Attempts to maintain a mouse-to-mouse infection for any period of time prior to the 4th adaptive passage were unsuccessful. Such infections were characterized by declining peaks, each peak being lower than that of the previous passage, and after the 3rd transfer infections were lost. After 4 adaptive passages, however, attempts proved successful, and blood from 4 mice which had been infected for 10 days produced a persistent low-grade parasitemia in the recipient mice. Transfers were made every 10th day through 9 passages covering a period of 90 days. The number of parasites was at no time high enough to allow accurate counting, and for this reason the series was discontinued. During the 4th mouse passage, blood from 2 mice was passed to chick embryos, and after 3 passages in the embryo, was transferred to the mouse. Three mice died as a result of inocula-

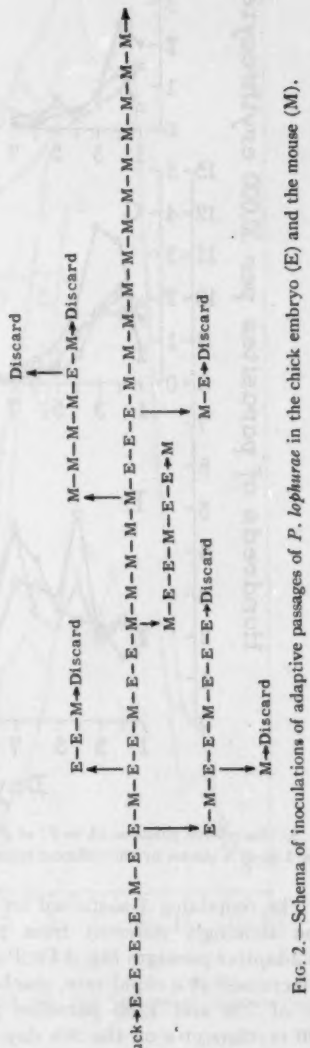

FIG. 2.—Schema of inoculations of adaptive passages of *P. lophurae* in the chick embryo (E) and the mouse (M).

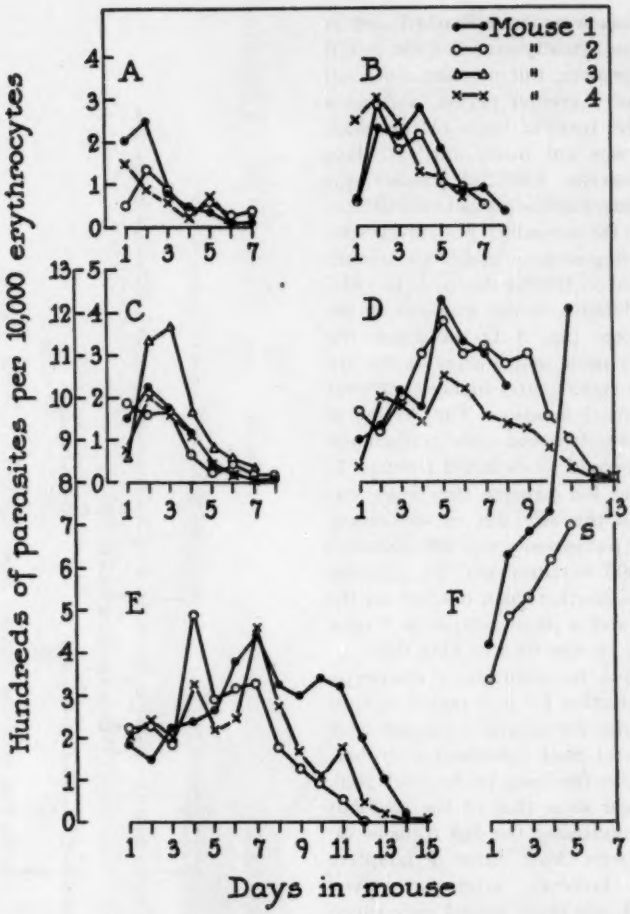

FIG. 3.—Successive passages (A to F) of *P. lophurae* in mice. The original source of the plasmodia (Mouse 1 to 4) is shown by the different types of lines.

tion. The remaining 2 sustained an infection strikingly different from previous adaptive passages (fig. 3 F). Parasites increased at a rapid rate, reaching peaks of 700 and 1200 parasites per 10,000 erythrocytes on the 5th day, at which time both mice were killed, and the blood was pooled and inoculated into infant mice. The parasite level of the donor mice was of such proportions that initial counts were possible in the recipients, and the course of infection could be followed with relative ease.

The number of mice infected through the 4th passage was small, and the graphs of figures 3 and 4 comprise the total numbers infected in most passages. The course of infection for the 1st, 2nd, 5th, and 6th passages derived from the 5th adaptive embryo-mouse passage

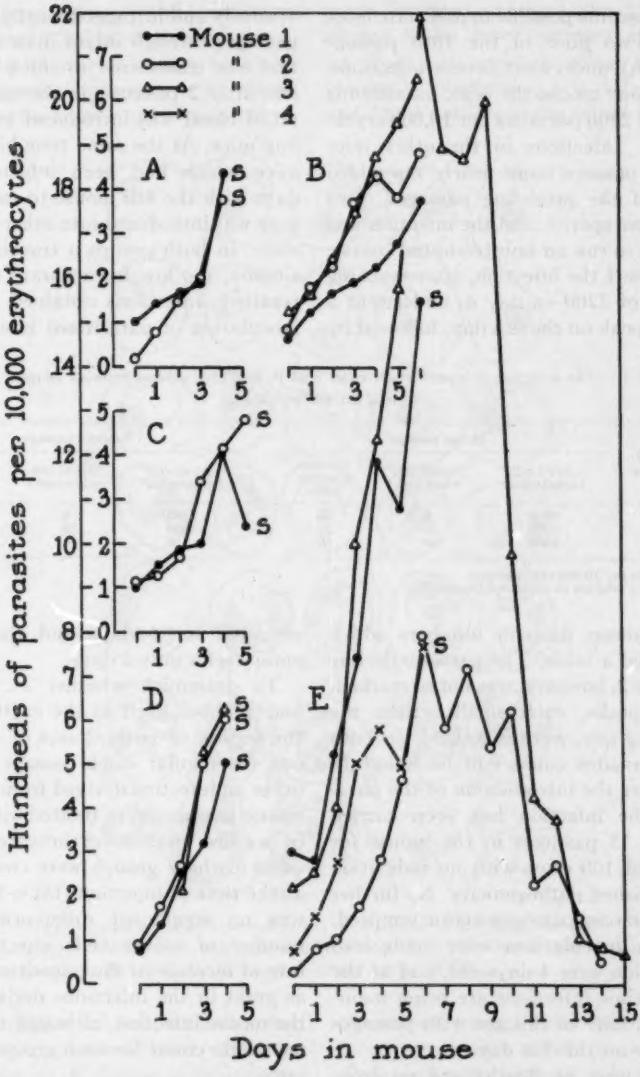

FIG. 4.—The course of infections of the 1st, 2nd, 5th, 6th, and 10th mouse-to-mouse passages of *P. lophurae*.

was relatively similar (fig. 4 A, B, C, D) in all, with the exception of one mouse in the 2nd passage, and was characterized by a gradual rate of increase until the time of transfer. Subsequent infections until the 10th passage were also similar. After the 3rd passage the amount of blood given each was reduced to 0.05 ml.,

and it became possible to inoculate more mice. Two mice in the 10th passage (fig. 4 A) underwent severe infections, and in one mouse the peak parasitemia reached 2200 parasites per 10,000 erythrocytes. Infections in the other mice of this passage more nearly resembled those of the preceding passages. Two mice were spared, and the infection was allowed to run an uninterrupted course. In mouse 1 the infection, after reaching a peak of 2200 on day 6, underwent a second peak on the 9th day, followed by a precipitous drop in numbers which resembled a crisis. The parasite decline in mouse 2, however, was not so marked, and 3 peaks, each smaller than the preceding one, were sustained. In both mice parasites could still be found 16 days after the introduction of the parasites. The infection has been carried through 15 passages in the mouse for a total of 100 days with no indication of diminished pathogenicity. No further embryo-mouse passages were attempted. Recently inoculations were made into mice which were 4 days old, and at the present time infections are being maintained in mice of this age with passages regularly on the 7th day.

There were no deaths and no gross pathological changes either external or internal in mice infected with *P. lophurae.*

In adult mice infections of a transient nature were observed only after massive doses of parasites were introduced intravenously and intraperitoneally. After 10 passages through infant mice the infection was transferred to chick embryos, and after 2 passages in the embryo 0.4 ml of blood was introduced into weanling mice. At the same time blood from mice which had been infected for 7 days with the 8th mouse-to-mouse passage was introduced into other weanling mice. In both groups a transient parasitemia, too low for accurate counting, resulted. Infections obtained from the inoculation of parasitized mouse blood persisted for 6 days and those from embryos for only 2 days.

TABLE 1.—*The infection of mouse erythrocytes with* P. lophurae *derived from an adapted murine and embryo infection.*

Day of infection	Mouse passage			Embryo passage		
	Avial cell parasitemia*	Mouse cell infection*	Percent of mouse cells	Avial cell parasitemia*	Mouse cell infection*	Percent of mouse cells
2	2000	0	0	2300	0	0
3	2700	15	26	4800	28	31
4	6900	40	16	0	—	—

* Parasites per 10,000 erythrocytes.
Counted in relation to avian red blood cells.

To determine whether *P. lophurae* had adapted itself to the erythrocytes, the serum, or both, 2 sets of embryos, one the regular stock passage and the other an infection derived from the 10th mouse passage, were treated with 0.2 ml of washed mouse erythrocytes. Infections in the 2 groups were comparable at the time of injection (table 1). There was no significant difference in the number of mouse cells infected. The rate of increase of the parasites was not as great in the infections derived from the mouse infection, although the mean merozoite count for each group was the same.

Morphology

In considering the morphology of *P. lophurae* in the mouse it was necessary to study the parasites from the initial and final passages, and in addition those

parasites from the early and later stages of the infection in the established strain. During the adaptive passages, either by embryo-mouse alternation or in mouse passage alone, there was a decided tendency on the part of the parasites toward assuming more and more the characteristics of *P. lophurae* in the bird. The early trophozoites were similar in all infections, and the characteristic ring froms generally associated with mammalian malaria were absent (plate I, fig. 1–3; plate III, fig. 16–18). Banding was common in initial infections, while in later passages the parasites tended to be more compact (plate I, fig. 9–15) although banding was occasionally observed (plate III, fig. 26 and 28). Parasites in the initial through the second passage retained the vacuolated appearance which is peculiar to *P. lophurae* in the duck (plate III, fig. 14). Occasionally even in the established strain parasites with vacuolated cytoplasm were seen (plate I, fig. 20). In initial passages a large number of degenerate parasites were seen, particularly before the completion of the first

PLATE 1.—The morphology of *P. lophurae* in the erythrocytes of the infant mouse.
1–3: Young trophozoites. 4–10: Slightly older trophozoites. 11–16: Uninucleate schizonts. 17–33: Segmenting parasites. 34 and 35: Gametocytes. Camera lucida. ×1500.

PLATE II.—A comparison of the morphology of *P. lophurae* in the chick embryo, in initial and established mouse infections.

A1–13: Initial mouse infections. B1–13: Parasites in the 10th mouse-to-mouse passage. C1–13: Similar forms in the chick embryo erythrocyte. Parasites are drawn without the host cell to facilitate comparison. Camera lucida. ×1500.

TABLE 2.—*A comparison of the sizes in microns of cells and parasites in 4- and 12-day infected mice.*

Erythrocytes infected with	4 days		12 days	
	Cells	Parasites	Cells	Parasites
No parasites	6.85±0.1		5.5 ±0.04	
Young trophozoites	7.00±0.1	1.17	5.80±0.1	1.11
Parasites with:				
1 nucleus	7.00±0.1		5.9 ±0.1	
4 or more nuclei	7.2 ±0.12	4.92	5.4 ±0.13	3.92

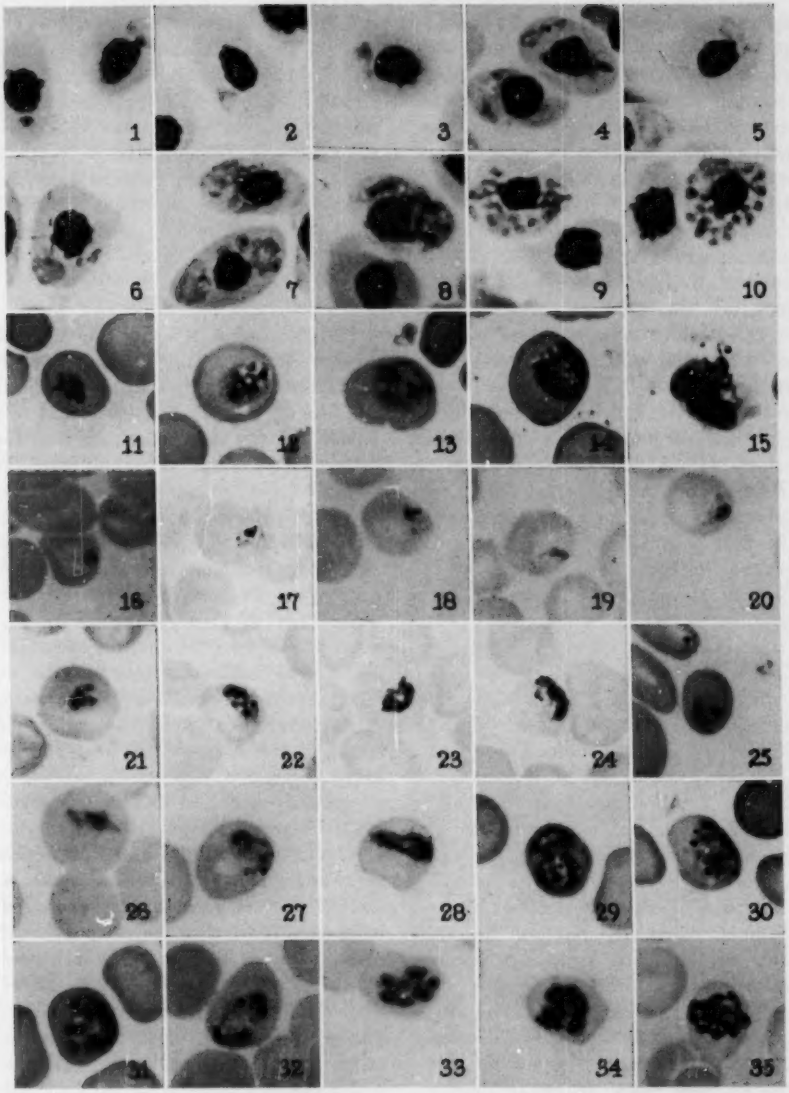

PLATE III.—Photomicrographs of *P. lophurae* in chick embryo erythrocytes (fig. 1–10), in the initial mouse infection (fig. 11–15), and in the 10th mouse passage (fig. 16–35). ×1500. Photographs by Mr. J. A. Carlile.

sporulation (Plate II, fig. A10 and A12; plate III, fig. 14 and 15). These parasites were marked by a diffuse pink-staining nucleus especially in the more mature forms, although a certain proportion of schizonts were apparently normal throughout the period (plate II, fig. A13). Pigment granules were large, dark, and were not arranged in any distinct pattern. As early as the first nuclear division the pigment of some parasites was clumped (Plate II, fig. A7), and this was nearly always in evidence after the third division of the nucleus.

In the established mouse strain the pigment granules were a finer, lighter color (plate I). The nucleus was more compact and stained more darkly. Fewer degenerate parasites were seen prior to the first sporulation, and there was a gradual decrease after that time. The sizes of parasites and host cells were obtained by direct measurement of uninfected and infected cells and of parasites at different stages of development, both at 4 days and at 12 days after infection of the 10th mouse passage. During the period covered there was a reduction in the size of both normal and infected erythrocytes from $6.85 \pm 0.1\mu$ to $5.82 \pm 0.1\mu$, and a concomitant decrease in the diameter of the mature schizonts from 4.92μ to 3.92μ (table 2). There was no significant enlargment of the host cell at any stage of the parasite's development, and the maximum area of the cell occupied by the parasite was about three-fourths. The merozoite mean was determined by counting the merozoites of 75 segmenting parasites in chick embryos and in mice infected for 4 and 12 days. The mean merozoite count in embryos was 15.3 ± 0.04, in 4-day mice, 9.8 ± 0.2, and in 12-day mice, 7.5 ± 0.04.

Gametocytes were absent from all infections except in one mouse of the established strain. One parasite was free in the cytoplasm and may have represented a degenerate asexual form (plate I, fig. 34), while the other was in an erythrocyte and was tentatively classified as a macrogametocyte (plate I, fig. 35). Gametocytes were numerous in embryo infections whether derived from the initial mouse infection or final adaptive strain.

DISCUSSION

Initial infections of the mouse with *P. lophurae* are similar in many respects to *Plasmodium falciparum* infections in the monkey.[6] Both hosts manifested a strong natural resistance necessitating little, if any, acquired immunity in the elimination of the parasites. Gametocytes were demonstrated in neither infection. Unlike *P. falciparum*, however, *P. lophurae* has passed to a mammal from an avian host, the chick embryo, which has practically no innate immunity and no demonstrable acquired immunity. Conditions were such, therefore, that the few surviving parasites in the mammalian host could be transferred to the nonresistant host and allowed to produce a population in which a certain proportion had an enhanced ability toward survival in the mouse. This process of adaptation by selection was, however, seriously hampered by the inability of the avian erythrocytes to survive for any period of time in the mouse. The loss of avian cells was in many instances complete in 24 hours. With the erythrocyte destruction there was a concomitant loss of parasites, and as a result only those parasites which segmented prior to the host cell destruction were potential invaders of mouse cells. When avian erythrocytes were still present in appreciable num-

6. Taliaferro, W. H. and Taliaferro, L. G. 1934, Am. J. Hyg. **19**: 318–334.

bers after 24 hours, there was a marked reinvasion negating the favorable disposition of the host animal to the foreign inoculum. The reinvasion of avian erythrocytes was in certain mice of such proportions as to double the parasitemia. In no instance was the initial mouse cell infection higher than 4%. It would seem then that there is a resistance on the part of the mouse cell which when coupled with the presence of an adequate number of nonresistant cells resulted in reinvasion of the latter.

Once the parasite had adapted itself to the point that successful mouse-to-mouse passages could be effected, the embryo-mouse passage was no longer the method of choice. Rapid passage would in all probability lessen the selection, and as a result succeeding passages would not demonstrate the degree of adaptation that was seen in the embryo-mouse passage.

In the mouse, as in the bird, age has a decided influence upon infection with *P. lophurae*. The process of avian cell destruction is augmented in older mice, reducing the chances of murine cell invasion. Weanling mice receiving injections of parasites which had undergone 10 passages in infant mice also exhibited a strong resistance which almost completely suppressed infection. It has been found (unpublished data) that the erythrocytes of the mature mouse are fully as susceptible to infection as those of the infant. The most potent factor in the destruction of the parasites lies not in the erythrocyte, but in other defense mechanisms, such as the plasma or the macrophage system. Adaptation of the parasite, therefore, has been directed toward survival in the presence of these elements rather than invasion of the erythrocyte.

The extreme rarity of gametocytes may be laid to the effects of unfavorable environmental conditions present in the mouse. It is of interest to note that infections of chick embryos initiated from a murine strain are marked by the production of gametocytes in the 1st passage. Whether this reflects the presence or absence of exoerythrocytic stages[7] is impossible to surmise at this time, since blood induced phanerozoites have as yet not been demonstrated in *P. lophurea* infections.

SUMMARY

The intravenous inoculation of chick embryo blood heavily infected with *Plasmodium lophurae* into infant mice gave rise to a low grade parasitemia. By alternating embryo and mouse passages the course of infection in the mouse became modified in that greater peaks of parasite numbers were attained and the period of patent parasitemia was lengthened. It became possible after four such passages to pass the infection from mouse to mouse without any apparent diminution of viability on the part of the parasite. The establishing of *P. lophurae* in the mouse was accompanied by distinct morphological changes, with a tendency toward reassuming the characteristics seen in avian cell infections.

7. Lewert, R. M. 1950, Am. J. Hyg. 51: 178–194.

RESISTANCE OF LUNGS TO STREPTOCOCCI AS AFFECTED BY ASCORBIC ACID BLOOD LEVEL OR HISTAMINE ADMINISTRATION

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Resistance of the respiratory tract is an extremely important factor in determining whether or not an individual falls victim to the numerous respiratory tract pathogens with which he daily comes in contact. It must be granted that heightened virulence of the pathogen for the human respiratory tract may be a peculiar and at present an unmeasurable property of certain strains of parasites. However, it seems probable from epidemiological evidence that virulence of the pathogen is not as significant as alterations in resistance of the host in accounting for the spotty distribution of many of the more important and common respiratory tract infections. Furthermore, it is improbable that a single factor is responsible for this fluctuation in resistance. Attention directed to variations in nutritional factors, such as vitamin or protein intake, fails to give a cleancut correlation with the fluctuation in resistance in man necessary to account for the haphazard distribution of respiratory tract infections. The consideration of fatigue and loss of sleep likewise has contributed little to our knowledge of the fundamentals of this problem. Attempts to explain altered resistance of the respiratory tract, especially the lungs, in terms of the accumulation of excessive amounts of mucus with resulting atalectasis[1] or in terms of effects on the phagocytic cells of the respiratory tract[2] have been investigated with suggestive results. Regardless of the mode of action of excessive quantities of mucus in the respiratory tract, it seems quite true that mucus will lower resistance of the lungs when present in greater than physiological amounts. Several studies by the senior author and colleagues have been published on the possible effect of cold, alcohol, ether anesthesia,[3] and respiratory pattern[4] in lowering resistance by affecting the accumulation of mucin in the lower respiratory tract. Alcohol, as affecting the phagocytes and, thus, lowering resistance, has been investigated from two points of view, namely, (1) its effect in decreasing the permeability of the capillaries to leukocytes in the area of inflammation[5] and (2) its interference with motility and chemotaxis of leukocytes.[6] It is not yet possible to determine the importance of the mechanisms so far mentioned as affecting resistance of man to infectious diseases. It seems to be in order, however, to continue the search for additional factors which conceivably could operate to lower the resistance of man

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1. Coryllos, P. M. and Birnbaum, G. L. 1929, Arch. Surg. **18**: 190–241.
2. Gunn, F. D. and Nungester, W. J. 1936, Arch. Path. **21**: 813–830.
3. Nungester, W. J. and Klepser, R. G. 1938, J. Infect. Dis., **63**: 94–102.
4. Nungester, W. J., Klepser, R. G. and Kempf, A. H. 1942, J. Infect. Dis. **71**: 57–60.
5. Pickrell, K. L. 1938, Bull. Johns Hopkins Hosp. **63**: 238–260.
6. Klepser, R. G. and Nungester, W. J. 1939, J. Infect. Dis. **65**: 196–199.

to respiratory infections. Ultimately, it will be necessary to evaluate the importance of all mechanisms postulated as affecting resistance, in terms of data from various pertinent fields, anatomy, physiology, immunology, biochemistry, etc., for a given disease and its associated clinical conditions. Only then can relative significance be assigned to the various possible mechanisms for lowered resistance.

The work reported in this paper deals primarily with experiments in which attempts have been made to alter the resistance of the lungs to a test organism in guinea pigs or rats by either of two procedures, namely, (1) by changing the ascorbic acid level of the host and (2) by local administration of histamine. Resistance has been estimated primarily by the rate of disappearance of the test organism from the pulmonary tissue.

METHODS

The experimental procedure usually followed was to expose the animals (guinea pigs and occasionally rats) to an aerosol of the test organism. Some of the animals were given parenteral injections of freshly neutralized ascorbic acid, some were kept on a diet deficient in vitamin C, others were exposed to a histamine aerosol at the same time the test organisms were administered, and still others were given histamine intraperitoneally prior to the organism aerosol. The conditions aimed at altering the resistance of the lung will be defined more specifically later.

The aerosol chamber used for exposing the animals to test bacteria consisted of an autoclave 17 inches in diameter and 36 inches long, divided into two chambers 11 inches from the closed end by a brass door. This door had brazed into it eight containers for holding guinea pigs with their heads protruding and noses pointed toward the incoming aerosol. The aerosol was produced in a DeVilbiss No. 40 nebulizer modified so that dry air was mixed with the nebulized material. This served to lower the moisture content of the aerosol and to decrease the particle size. Appropriately placed flowmeters aided in producing aerosols with more or less constant characteristics.

The method of operating the exposure chamber was as follows: the animals were placed in holders with their heads pointing toward the nebulizer oulet. A small vacuum was established with the aid of a mechanical pump which exhausted to the outside of the building. A 24-hour broth culture of the organisms diluted 1:10 with saline solution (0.85% sodium chloride) or histamine solution was placed in the nebulizer. The nebulizer was then connected to the chamber and an aerosol was generated for a period of 15 minutes by passing air through the nebulizer at the rate of 12 L per minute, dry air being added at the rate of 12 L per minute. At the end of this time, the nebulizer was cut off and the number of organisms in the chamber reduced by continuing the flow of clean air through the unit for 20 minutes.

Following exposure in the chamber, the animals were removed, sacrificed at indicated intervals, and autopsied. The number of organisms and amount of histamine reaching the lungs of the animals were determined by two methods. The direct approach was to sacrifice two of the 8 animals immediately after exposure, and determine by plate count the number of organisms in the lung. The middle left lobe was removed, ground with sand, and appropriate dilutions plated. The amount of drug was estimated by assuming that the ratio of organisms to drug in the lung would be the same as that placed in the nebulizer.[3] The indirect approach consisted of using an air sampler of the liquid impinger type to determine the number of organisms in the air of the chamber. The sampler fluid employed was sterile brain heart infusion broth plus corn oil added as an antifoam. The sampler count is of importance primarily in determining the bacterial concentration in the chamber from experiment to experiment; this supplies a basis for comparison of cloud concentration of bacteria, which is only one factor determining the number of organisms which reach and stay in the lungs of the animals. The belief that the "biological sampler" is more discriminating than the mechanical samplers in current use is based on the consideration that the respiratory challenge of an animal depends on (1) number of organisms in the air, (2) their particle size, (3) tidal air of exposed animals, (4) loss of organisms by impingement on walls of chamber and air passages of animal, and (5) most significantly, the number of organisms retained in the lung. The mechanical samplers measure only the organisms present in a given volume of air. Nevertheless both types of measurements have been made in a great part of this work, and the number of organisms found in the lung at different intervals of time after exposure under various conditions have been expressed as percent of sampler count, to facilitate the comparison of data of different experiments.

RESULTS

Ascorbic acid blood levels.—A large number of guinea pigs (130) were exposed to aerosols of several strains of hemolytic streptococci. Ascorbic acid blood levels were varied by intraperitoneal administration of the freshly neutralized acid or by restricting the dietary intake of the vitamin. Ascorbic acid levels were determined by the method of Roe and Kuether[7] using the Beckman spectrophotometer. The rate of disappearance of the test organism from the lungs of guinea pigs was determined by sacrificing animals at intervals during the first 24 hours after exposure and also at 48 and 72 hours. With the test organisms employed, it was not possible to find any correlation between the rate of disappearance of the organism from the lung and high or low ascorbic acid blood levels or ascorbic acid intake. In these experiments the initial content of streptococci in the entire lung after exposure was of the order of 100,000 organisms. After 24 hours, 12 of 23 animals had no streptococci, and at 48 hours 26 of 42 were free of the organisms. These results were somewhat surprising, since previous work in this laboratory had shown that phagocytosis in vitro was low when the ascorbic acid blood level was down. On this basis it had been anticipated that guinea pigs with low ascorbic acid intake would probably not clear the lungs of the test organisms as rapidly as normal animals. The data accumulated in this study did not support this hypothesis. Furthermore, animals exposed to the test organism and sacrificed 6 or 7 days later did not develop pneumonia except in a very few instances, less than 5%. There was no indication of any correlation between the development of pneumonia and ascorbic acid blood level among the few animals in which pneumonia did develop.

TABLE 1.—*Effect of ascorbic acid blood levels on rate of clearance of streptococci from lungs of guinea pigs.*

Hrs. after exposure at which animals were killed	Number of animals	Ascorbic acid		Streptococci $\frac{\text{lung}}{\text{sampler}} \times 100$
		Intake	Whole blood (mg/100 ml)	
0	12	High	0.86	8.1 %
	11	Normal	0.33	5.5 %
	6	Low	0.11	5.0 %
1	10	High	0.78	2.62%
	10	Normal	0.23	2.09%
	6	Low	0.11	2.69%
3	6	High	0.89	1.21%
	9	Normal	0.10	1.09%
	2	Low	0.21	1.64%
6	2	High	0.99	0.13%
	2	Normal		0 %
	2	Low	0.45	0.52%
10	4	High	1.06	0.04%
	4	Normal		0 %
	2	Low	0.09	0.10%

In the experiments reported, considerable difficulty had been encountered with chronic lung infections. In fact over 90% of the animals at one time showed some evidence of such disease in one or more lobes of the lung. At a later date the investigation on the role of ascorbic acid metabolism on lung resistance was repeated, using animals that were relatively free of lung disease.

7. Roe, J. and Kuether, C. 1943, J. Biol. Chem. **147**: 399–407.

The data (table 1) from these experiments not only support in general the earlier results, but also bring out two minor effects of ascorbic acid. High blood levels of ascorbic acid were obtained by intraperitoneal injection of freshly neutralized ascorbic acid, 50 to 100 mg daily for 2 to 5 days prior to aerosol challenge. Animals with low levels were kept on an ascorbic acid free diet. Normal animals were fed standard Rockland pellet and alfalfa hay diet. It was observed that about one-third more streptococci (8.1% of sampler count) reached the lungs in animals with high levels of vitamin C than in normal animals (5.5%) or those with low ascorbic acid levels (5.0%). On the other hand, animals with high blood levels cleared the lungs of streptococci within 10 hours despite a larger initial inoculation.

It has been suggested by Bessey and Lowry[8] that ascorbic acid values of whole blood are not as indicative of tissue levels as values determined on the leukocytes of blood. Analyses of both the blood and the peritoneal inflammatory exudate of 573 guinea pigs gave no evidence of a discrepancy between blood levels and inflammatory exudate levels* (table 2). With few exceptions, less than 5%, the exudate and blood were essentially parallel.

Histamine and Lung Resistance.—Since the recognized effect of histamine on capillaries is to increase their permeability, it might be expected that the administration of this drug as an aerosol would result in pulmonary edema. It is generally assumed, primarily on the basis of clinical experience, that edema of the lungs lowers their resistance to bacterial invasion. It is well recognized that histamine is present in normal tissue, particularly that of the lungs, as an intracellular constituent. When it escapes from the damaged cells, it may produce pathological effects quite unexpected from its quiescent behavior as an intracellular component. Histamine is also produced by various strains of bacteria in culture mediums.[9,10] It has been detected in nasal secretions of patients with the common cold.[11] The supposed effect of pyribenzamine[12,13] in altering the symptomatology of some of the upper respiratory infections designated as the common cold may be due

TABLE 2.—*Distribution of guinea pigs with indicated blood and exudate levels of ascorbic acid.*

Blood level	Exudate 0.0 to 0.1	0.11 to 0.5	0.51 to 1.0	1.01 to 1.5	1.5	Number of guinea pigs in each blood level group
0.0 to 0.1*	3	1	0	1	1	6
0.11 to 0.5	7	56	61	7	0	131
0.51 to 1.0	0	26	155	83	18	282
1.01 to 1.5	0	0	8	36	43	87
1.5	0	0	7	4	56	67
Totals	10	83	231	131	118	573†

* Values for ascorbic acid given in mg/100 ml.
† Similar findings with exudate levels were obtained in an additional 500 guinea pigs not included in this table, since blood samples were not analyzed.

* These determinations were made by Mr. D. Alonso.

8. Bessey, O. A., Lowry, O. H. and Brock, M. J. 1947, J. Biol. Chem. **168**: 197–205.
9. Kendall, A. I. and Gebauer, E. 1930, J. Infect. Dis. **47**: 261–266.
10. Eggerth, A. H. 1939, J. Bact. **37**: 205–222.
11. Troescher-Elam, E., Ancona, G. R. and Kerr, W. J. 1945, Am. J. Physiol. **144**: 711–716.
12. Brewster, J. M. 1947, U. S. Nav. M. Bull. **47**: 810–811.
13. Brewster, J. M. 1949, U. S. Nav. M. Bull. **49**: 1–11.

to its antihistaminic properties, regardless of whether one interprets this effect as part of a sensitization phenomenon or as a possible effect on histamine arising directly from the infectious process from bacteria or damaged tissue. These considerations give rise to the following question: Could small amounts of histamine lower the resistance of the lung parenchyma and so predispose to pneumonia if the histamine formed by bacteria in the nose, trachea, or bronchi, or liberated from damaged cells at these sites, reached the lung by one method or another?

There are other infectious processes in which histamine, if it lowers resistance, may play a role, as in gas gangrene, tuberculosis, pneumonia, etc. In pneumonia, for example, is the spread of the pneumonic process, once it has been initiated by whatever factor which lowers the resistance of the host in the first place, dependent in part on histamine effects? In experimental pneumonia in the rat,[14] the original lesion initiated by the intrabronchial injection of mucin and pneumococci is relatively small but rapidly spreads to involve an entire lobe. An inoculum of a normal lung with large numbers of organisms but without mucin produces pneumonia in very few animals. Mucin is an important initiating factor in this particular process; yet, once the process has been started, it spreads rapidly without the aid of this material. There is need for an adequate explanation for the continuation of the pneumonic process in this experimentally produced disease.

TABLE 3.—*Survival of streptococci in lungs of guinea pigs as affected by administration of histamine.*

Strep. no.	Time in days	Organism in aerosol control		Histamine with organisms in aerosol once		Histamine intraperitoneal		Histamine aerosol daily		Histamine intraperitoneal daily		Histamine in aerosol benadryl. Intraperitoneal (one injection)	
87	0	(8)*	23.0%†	(6)	9.0%			(8)	9.0%	(6)	45.0%	(6)	14.0%
	1	(8)	2.2%	(6)	121.0%			(9)	2016.0%	(4)	44.0%	(6)	0.0%
	2	(8)	2.2%	(6)	1133.0%			(9)	74844.0%	(5)	7.0%	(6)	1.0%
	3	(8)	0.0%	(6)	0.0%			(10)	38413.0%	(1)	33928.0%	(1)	17200.0%
										(4)	10.0%	(5)	0.0%
91	0	(4)	34.0%	(4)	45.0%								
	1	(4)	0.0%	(4)	216.0%								
	2	(4)	0.0%	(4)	223.0%								
	3	(4)	0.0%	(4)	33.0%								
73	0	(10)	19.0%	(29)	14.2%	(13)	13.0%						
	0.25	(8)	4.4%	(8)	0.7%	(10)	1.0%						
	1	(8)	0.0%	(24)	149.0%	(11)	0.0%						
	2			(4)	0.0%	(4)	0.0%						

* Number of animals used.

† $\frac{\text{Lung count}}{\text{Sampler count}} \times 100$.

The results (table 3) obtained in experiments using histamine to lower resistance were quite constant. When small quantities of the drug, estimated to be 0.5γ, were administered as an aerosol to guinea pigs along with an aerosol of the test organism, *Streptococcus hemolyticus* type C, a marked change in the rate of disappearance of the organisms from the lung was noted. Instead of the drop in count per lung to nearly zero in 8 to 10 hours, as seen in the controls of this experiment, an early drop occurred in the first 6 hours, followed by a rise. The number of test organisms present in the lungs at 24 hours was often nearly double the number found immediately after exposure to an aerosol. By 48 hours, the streptococci

14. Nungester, W. J. and Jourdonais, L. F. 1936, J. Infect. Dis. 59: 258–265.

had usually disappeared from the lung, If the animals received a second exposure to an aerosol of histamine 24 hours after the initial streptococcus and histamine aerosol, the number of organisms in the lungs remained high. As will be noted in table 3, three daily exposures of histamine aerosol resulted in a high bacterial content in the lungs for a three day period. Histamine given intraperitoneally instead of by aerosol had little or no effect on the rate of disappearance of organisms as compared with controls.

If histamine effects were neutralized in vivo by the injection into the guinea pig of an antihistamine, such as benadryl (10 mg given intraperitoneally 15 minutes before exposure to the aerosol of streptococci and histamine), the rate of disappearance was the same as that occurring in animals not given histamine. Benadryl prevents the effect of histamine responsible for the lowering of pulmonary resistance. It is interesting to note that the rat peculiarly resistant to the effects of histamine removed the test organism as rapidly following an exposure to an aerosol of histamine and streptococci as after a challenge with an aerosol of the streptococci alone.

The mechanism by which histamine lowers the resistance of the lungs to streptococci is probably not, as originally postulated, the production of pulmonary edema. Attempts to demonstrate edema by increased ratios of lung to body weight following the administration of a histamine aerosol were not successful. There was too much fluctuation in this ratio within both groups of animals. Using a stethoscope breath sounds resembling bubbling rales were heard in animals receiving histamine. Histological examinations* were made of the lungs of animals exposed to a histamine aerosol and of the lungs of control animals. The animals were killed, the trachea tied off, and the lungs fixed in 10% formalin for 24 to 48 hours with constant shaking. The results showed some evidence of edema in 6 of 9 guinea pigs killed within 15 minutes after exposure to an aerosol of histamine. In another group of guinea pigs killed 24, 48, and 72 hours after exposure to histamine aerosol, 6 of 9 animals showed the presence of edema in amounts increasing with the lapse of time. One of the two animals subjected to an aerosol of physiological saline revealed a small amount of seromucous exudate in the bronchi; and 4 of 7 animals receiving an aerosol of the organism and physiological saline showed slight edema. There was no edema observed in 4 animals given benadryl before exposure to the histamine aerosol. In interpreting these results it is necessary to recall that edema of the lungs is often noted in animals that have been sacrificed. It is doubtful that the small amount of edema possibly produced in the lungs by the histamine is the significant factor responsible for the delayed clearance of streptococci. Since some edema was noted in animals treated with an aerosol of saline solution and bacteria, and these animals did clear the organisms rapidly from the lung, it might appear that histamine lowered resistance by some other mechanism than the production of edema. The possibility that histamine might inactivate the cilia of the bronchi and trachea was investigated. The tracheas of several guinea pigs were excised, opened, and fixed flat on a slide, then examined at approximately 400× magnification. The addition of histamine from a capillary pipette, in amounts of from 10 to 100γ/ml, produced no adverse effect during the 10 minute period of observation of ciliary action.

* We are indebted to Dr. Carl V. Weller for these findings.

SUMMARY

The rate of clearance of streptococci from the lungs of guinea pigs following exposure to an aerosol of the test organism has been investigated. No marked difference was noted between groups of animals with high blood levels of ascorbic acid and others with low levels. One administration of 0.5γ of histamine to the animals via an aerosol decreased the rate of disappearance of streptococci from the lungs. Daily exposure of the animals to histamine resulted in a marked increase in streptococcal content of the lungs. Benadryl neutralized this effect of histamine. Some edema was noted in lungs of animals exposed to histamine as well as in the control groups. It is doubted that edema is a significant factor in the lowered resistance of animals exposed to histamine. The cilia of the trachea were not affected by local administration of histamine.

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